Expression and regulation of cyclooxygenase 2 in colonic epithelial cells

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EXPRESSION AND REGULATION OF CYCLOOXYGENASE 2 IN COLONIC EPITHELIAL CELLS

submitted by

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for the degree of Ph.D.

of the University of Bath

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ABSTRACT

The gastro-intestinal tract is the commonest site of internal malignancy and also susceptible to the chronic inflammatory bowel diseases, Crohn's disease and ulcerative colitis. The intestinal epithelium represents a complex, immunologically active, barrier to the large number of antigens present in the gut. Intestinal epithelial cells can present antigens, take part in the sophisticated regulation of intestinal inflammation, as well as potentially undergoing malignant change.

Cyclo-oxygenase (COX) is the enzyme which synthesises prostaglandins and thromboxanes and COX-2 is its inducible isoform. COX-2 is up-regulated in the inflammatory bowel diseases as well as in the majority of colorectal carcinomas. There is increasing evidence that COX inhibition protects against colorectal carcinoma, although the role of COX-2 in inflammatory bowel disease is less clear. COX-2 is known to be an important enzyme in the context of inflammation and oncogenesis and is highly regulated.

This thesis aimed to explore the induction of COX-2, and its regulation, in intestinal epithelial cells. In particular, it concentrated on the intra-cellular signalling pathways involved in its regulation. To pursue these aims HT-29 cells were used as an experimental model of intestinal epithelial cells with supportive work being performed in the Caco-2 cell line.

In intestinal epithelial cells, COX-2 was induced by the Th1 pro-inflammatory cytokines TNFα and IL-1. Cytokine induced COX-2 expression could be complexly regulated at the level of mRNA transcription and stability, COX-2 protein production, as well as the generation of COX-2 dependent products such as PGE_2. It could be regulated by its products such as PGE_2 as well as cyclopentenone prostaglandins which are PPARγ agonists. It was also regulated by IFNγ in opposing ways at the level of COX-2 mRNA compared to downstream protein and PGE_2 production. The Th2 cytokines IL-4 and IL-13 inhibited COX-2 expression consistently at all points.

TNFα and IL-1α activated the PI 3-Kinase signalling pathway in intestinal epithelial cells, as well as activating the three major MAP Kinases. PI 3-Kinase activation appeared to inhibit COX-2 expression although conflicting evidence was obtained using two different PI 3-Kinase inhibitors. It is proposed that PI 3-Kinase mediates the inhibitory actions of IL-4 and IL-13 on COX-2, as it is shown that these cytokines also activate PI 3-Kinase in this system. The cytokine induction of COX-2 expression and activity appears to be dependent on ERK1/2 and p38 MAP Kinase activation by TNFα and IL-1α. It is also demonstrated that TNFα and IL-1α can phosphorylate and inactivate GSK-3α/β and that this process is dependent on both PI 3-Kinase and ERK1/2 MAP Kinase which act synergistically in this context.

This thesis demonstrates the sophisticated mechanisms involved in regulating COX-2 in the intestinal epithelium. It also highlights the importance of the PI 3-Kinase and MAP Kinase signalling pathways in the epithelial response to inflammatory stimuli.

To

Jane

&

Grace

ACKNOWLEDGEMENTS

I have looked forward to typing this page as it marks the end of the work on my thesis and because so many people deserve thanks. A PhD may be an individual work, but there is much help and support along the way which takes many shapes and forms.

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CONTENTS

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ABBREVIATIONS

5-ASA	5-Aminosalicylic Acid
AA	Arachidonic Acid
Ab	Antibody
AcD	Actinomycin D
ADP	Adenosine Diphosphate
ANCA	Anti Neutrophil Cytoplasmic Antibody
ANOVA	Analysis of Variance
AP	Activator Protein
APC	Adenomatous Polyposis Coli
APS	Ammonium Persulphate
ARE	Adenylate Rich Element
ASCA	Anti *Saccharomyces Cerevisiae* Antibody
ATF	Activating Transcription Factor
ATP	Adenosine Triphosphate
AUF-1	AU-rich element/poly(U) binding factor 1
BSA	Bovine Serum Albumin
BTK	Bruton's Tyrosine Kinase
Ca^{2+}	Calcium^{2+} ions
Caspases	Cascade of aspartate specific cysteine proteases
C-C	Cysteine-Cysteine motif
CD	Crohn's Disease
CDn	Cluster of Differentiation

cDNA	Complementary Deoxyribonucleic Acid
CHX	Cycloheximide
CINC	Cytokine Induced Neutrophil Chemoattractant
ConA	Concanavalin A
COX	Cyclo-oxygenase
$cPLA_2$	Cytosolic Phospholipase A_2
cpm	Counts per minute
CRC	Colorectal Carcinoma
CRE	cAMP Response Element
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'chloro)tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
C-X-C	Cysteine – X– Cysteine motif
DAG	Diacylglycerol
dCTP	deoxy Cytosine Triphosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMBA	7,12-di-methylbenz[α]-anthracene
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DSS	Dextran Sodium Sulphate
DTT	Dithiothreitol
EBSS	Eagles Balanced Salt Solution
EDTA	Ethylenediaminetetraacetic Acid
EET	Epoxyeicosatetraenoic Acid
ELISA	Enzyme Linked Immunosorbent Assay

EMEM	Eagles Minimal Essential Medium
ENA	Epithelial Cell-Derived Neutrophil-Activating Peptide
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescent Activated Cell Sorting
FADD	Fas Associated Death Domain
FAP	Familial Adenomatous Polyposis
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
FKHRL	Forkhead Related Ligand
FLAP	5-Lipoxygenase Activating Protein
FLICE	Fas Associated Death Domain-like Interleukin-1β Converting Enzyme
fMLP	f-Methionine-Leucine-Phenylalanine
FRAP	FKBP12-Rapamycin-Associated Protein
GAS	Gamma Response Element
GRO	Growth Related Gene Product
GSK	Glycogen Synthase Kinase
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HBSS	Hank's Balanced Salt Solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HETE	Hydroxyeicosatetraenoic Acid
HLA	Human Leukocyte Antigen
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HPETE	Hydroperoxyeicosatetraenoic Acid
HPLC	High Performance Liquid Chromatography

hsp	Heat Shock Protein
HUVECs	Human Umbilical Vein Endothelial Cells
IBD	Inflammatory Bowel Disease
ICAM	Inducible Cell Adhesion Molecule
ICE	IL-1β Converting Enzyme
IE	Immediate Early
IEC	Intestinal Epithelial Cell
IEL	Intraepithelial Lymphocyte
IFN	Interferon
IFNGR	Interferon Gamma Receptor
Ig	Immunoglobulin
IGF	Insulin Like Growth Factor
IKK	Inhibitor of κB Kinase
IL	Interleukin
IL-1R	Interleukin-1 Receptor
IL-1Ra	Interleukin-1 Receptor Antagonist
IL-1RAcP	Interleukin-1 Receptor Accessory Protein
iNOS	Inducible Nitric Oxide Synthase
IP-10	Interferon-Inducible Protein 10
IRAK	Interleukin-1 Receptor Associated Kinase
IRF	Interferon Regulatory Factor
IRS	Insulin Receptor Substrate
ISRE	Interferon Stimulated Response Element
ITF	Intestinal Trefoil Factor
IκB	Inhibitor of κB

JAK	Janus Kinase
JNK	c-*jun* N-terminal Kinase
kb	Kilo-base
kDa	Kilo-Dalton
K-ras	Kirsten-ras
LOH	Loss of Heterozygosity
LOX	Lipoxygenase
LPMC	Lamina Propria Mononuclear Cells
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
LTα	Lymphotoxin α
LY294002	Specific PI 3-Kinase inhibitor
mAb	Monoclonal Antibody
MAP Kinase	Mitogen Activated Protein Kinase
MAPKAPK	Mitogen Activated Protein Kinase Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MCP	Monocyte Chemotactic Protein
MDA	Malondialdehyde
MEK	MAP Kinase ERK Kinase
MHC	Major Histocompatibility Class
MIP	Macrophage Inhibitory Protein
MKK	Mitogen Activated Protein Kinase Kinase
MLH	Human MutL homologue
MOPS	3-[N-morpholino]propane-sulphonic acid

MORT	Alternative name for FADD (Fas Associated Death Domain)
mRNA	Messenger Ribonucleic Acid
MSH	Human MutS homologue
MSI	Microsatellite Instability
mTOR	Mammalian Target of Rapamycin
MWt	Molecular Weight
Myc	Proto-oncogene from the avian myelocytomatosis virus
NF-IL-6	Nuclear Factor Interleukin-6
NFκB	Nuclear Factor κ B
NIK	Nuclear Factor κ B Inducing Kinase
NO	Nitric Oxide
NSAID	Non-Steroidal Anti-Inflammatory Drug
OD	Optical Density
PAF	Platelet Activating Factor
PD98059	Selective MEK1/2 inhibitor
PDGF	Platelet Derived Growth Factor
PDK	Phosphatidylinositol (3,4,5) Triphosphate Dependent Kinase
PG	Prostaglandin
PGHS	Prostaglandin GH Synthase
PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
PH	Pleckstrin Homology
PHA	Phytohaemagglutinin
PI	Phosphatidylinositol
PI 3-Kinase	Phosphatidylinositol 3-Kinase
PKB	Protein Kinase B

PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol-12-Myristate-13 Acetate
PMN	Polymorphonuclear Neutrophil
PMS	Gene Family involved in HNPCC
PMSF	Phenylmethylsulphonyl Fluoride
PPAR	Peroxisome-Proliferator Activated Receptor
PPRE	Peroxisome Proliferator Response Element
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and Tensin Homologue Deleted from Chromosome 10
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
RER	Replication Error
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid
ROM	Reactive Oxygen Metabolite
RTK	Receptor Tyrosine Kinase
RXR	Retinoic Acid Receptor
SB203580	Specific p38 MAP Kinase inhibitor
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SH	Src Homology
SHIP	SH2 containing Inositol 5' polyphosphatase

SODD	Silencer of Death Domains
$sPLA_2$	Secretory Phospholipase A_2
SSC	Saline-Sodium Citrate Buffer
SSPE	Saline-Sodium Phosphate-EDTA Buffer
STAT	Signal Transducer and Activator of Transcription
TACE	Tumour Necrosis Factor α Converting Enzyme
TATA	Adenine Thymine rich promoter sequence
TBS	Tris Buffered Saline
TCF	T cell Factor
TECK	Thymus-Expressed Chemokine
TEMED	N, N, N',N'-tetramethylethylene diamine
TGF	Transforming Growth Factor
Th1	T Helper 1
Th2	T Helper 2
TNBS	Trinitro benzene sulphonic acid
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor α Receptor
TPA	Phorbol Acetate
TRADD	Tumour Necrosis Factor α Receptor Associated Death Domain
TRAF	Tumour Necrosis Factor α Receptor Associated Factor
TXA_2	Thromboxane A_2
UC	Ulcerative Colitis
UTR	Untranslated Region
UV	Ultraviolet
Wnt	Contraction of Wingless and Int

Single letter amino acid codes

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln
R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

1 INTRODUCTION

1.1 Clinical Background

1.1.1 The Normal Intestine

The gastrointestinal tract provides a major interface between the body and the environment. It represents the largest collection of immune cells in the body and is exposed to an extremely diverse array of antigens and pathogens to which it must respond appropriately. Not only must this immunological response be tightly controlled, but so too must the high levels of cell turnover present throughout the length of the gastrointestinal tract.

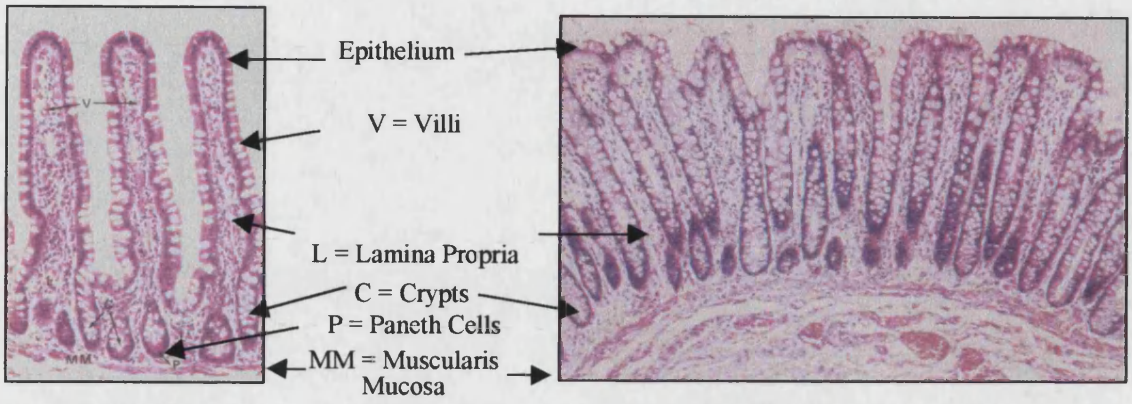
Progressing from proximal to distal in the gastrointestinal tract one goes from the mouth and pharynx through the oesophagus to the stomach. At the distal end of the stomach, the pylorus marks the beginning of the duodenum which is the first part of the small bowel or small intestine. This is followed by the jejunum and ileum with the ileocaecal valve marking the junction between the small and the large intestine or colon whose distal margin is the anal verge.

Moving from a macroscopic to a microscopic viewpoint, there is general pattern shared by the length of the intestine with specialised differences between each area. Starting from the lumen of the bowel there is an initial epithelial layer consisting of a

monolayer of intestinal epithelial cells (IECs) and scattered intraepithelial lymphocytes (IELs) with the basement membrane beneath. These form a membrane which is impermeable to all but the smallest molecules. This epithelial layer is replaced in its entirety every three to five days. Below this is the lamina propria, containing stromal cells and occasional lymphocytes as well as a rich vascular and lymphatic network, and delineated by the muscularis mucosa. These components make up the intestinal mucosa and are arranged architecturally into villi and crypts in the small intestine as opposed to straight tubular glands in the colon (see Figure 1.1). Below the mucosal layer is the submucosa, followed by an inner circular and outer longitudinal muscle layer. Finally there is the loose connective tissue of the serosal surface. Throughout the intestine there are occasional distinct lymphoid aggregates in the lamina propria such as Peyer's patches.

The immunological function of the intestine as a whole represents a highly sophisticated interplay between the large number of lymphocytes present and the intestinal epithelial cells. Indeed the normal intestinal mucosa can be said to be chronically inflamed, with this state of "physiological" inflammation being highly regulated (Dwinell and Kagnoff, 1999). The intestinal epithelial cells play an integral role within this process, being in a position to always be the first cells exposed to any lumenal component, and being able to generate and transmit numerous signals.

The repertoire of known intestinal epithelial cell (IEC) responses is large and continually expanding (Kagnoff and Eckmann, 1997). These include:



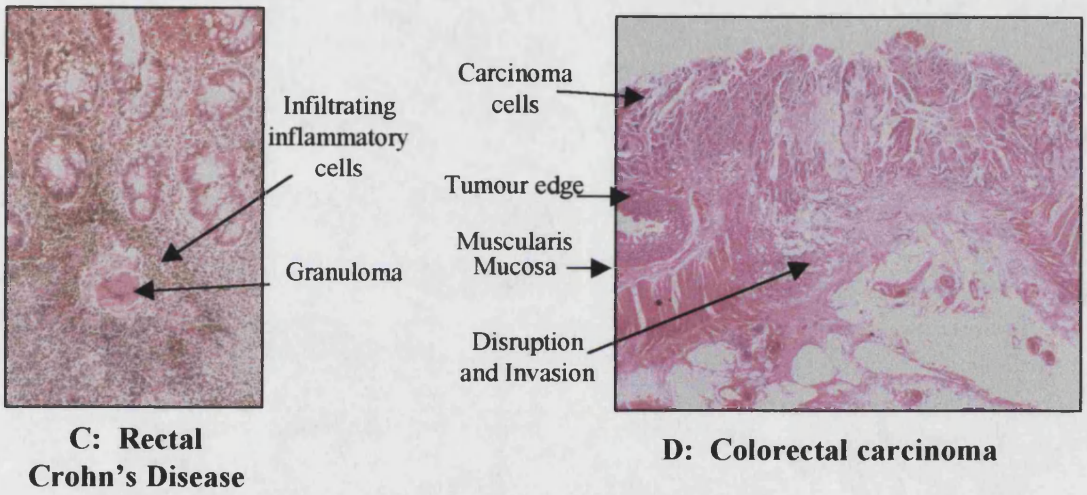


Figure 1.1: ***Histology of the intestine in health and disease***
Photomicrographs of the normal small (**A**) and large (**B**) intestine. **C**. Crohn's disease of the rectal mucosa showing massive infiltration by inflammatory cells and a characteristic granuloma. **D**. An adenocarcinoma of the colon showing epithelial proliferation, disruption of the normal architecture and invasion into deeper tissue. From Functional Histology by Wheater, P.R., Burkitt, H.G., Daniels, V.G. Churchill Livingstone

- ***Secreted epithelial products***: Stimulated IECs have been shown to secrete a wide variety of C-X-C chemokines (e.g.: IL-8, GROα-γ and ENA-78) and C-C chemokines (e.g.: MCP-1, MIP-1α and RANTES). They are also capable of releasing other pro-inflammatory cytokines such as TNFα and IL-1, and this seems to be in preference to immunoregulatory cytokines such as IL-4 and IFNγ implying a function in inducing rather than regulating an immune response (Jung *et al.*, 1995; Yang *et al.*, 1997).
- ***Antigen presentation***: IECs are capable of expressing MHC class II as well as class I molecules on their cell surface and have been shown to act as antigen presenting cells to T-cells in vitro (Hoang *et al.*, 1992).
- ***Cell surface receptor expression***: IECs have receptors to several pro-inflammatory (e.g.: TNFα and IL-1), immunoregulatory (e.g.: IFNγ, IL-2, IL-4) and chemotactic cytokines expressed on their surface (Kolios *et al.*, 1999).
- ***Cell adhesion molecule expression***: Stimulated IECs can up-regulate ICAM-1 which acts as a receptor for the β2 integrins expressed on PMNs and lymphocytes (Huang *et al.*, 1996).
- ***Expression of enzymes involved in the inflammatory response***: Enzymes such as iNOS and COX-2 have been shown to be induced in inflammation and iNOS can be induced in IECs (Kolios *et al.*, 1998). This work will concentrate on the induction and regulation of COX-2 by intestinal epithelial cells.

1.1.2 Inflammatory Bowel Disease

The inflammatory bowel diseases, comprising Crohn's Disease (CD) and Ulcerative Colitis (UC), are chronic relapsing and remitting conditions of the gastrointestinal tract. These conditions are predominantly diseases of the western world whose aetiology is unknown. However there is increasing evidence of a genetic component to host susceptibility, an environmental component most clearly seen as an important role of host intestinal microflora, and an aberrant or inappropriate immune response which may be intrinsically related to an associated gene defect (Fiocchi, 1998). Such an inappropriate immune response can have extra-intestinal manifestations such as in the skin, joints or eyes.

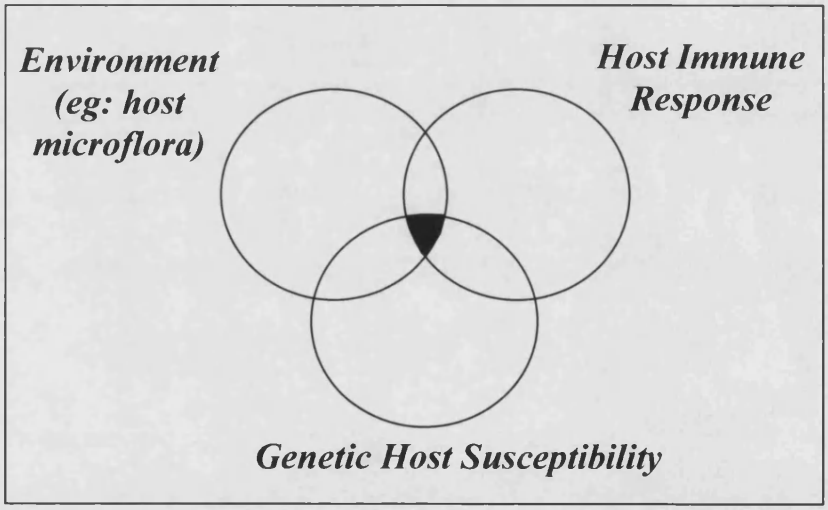


Figure 1.2: ***Aetiological factors in Inflammatory Bowel Disease***
Representation of the genetic, environmental and immunological components to the aetiology of IBD. There is no one clear agent and there is likely to be a complex interaction between many different factors

Although the precise aetiological mechanism is unknown the result is a chronic inflammatory process which commonly presents with bloody diarrhoea, can result in scarring and, if occurring in the colon for more than a decade can predispose to

colorectal carcinoma (Bansal and Sonnenberg, 1996). These generally descriptive features are shared by the two conditions, and indeed Crohn's Disease and Ulcerative Colitis may be indistinguishable in 10% of cases.

Also, from a historical point of view, the treatments for both Ulcerative Colitis and Crohn's Disease have been very similar, based on the use of 5-aminosalicylic acid (5-ASA) or non-specific immunosuppression with steroids. 5-ASA was first used in the 1940's and its precise mechanism of action remains unknown. However it has been shown, *in vitro*, to have a number of potentially beneficial effects in the context of IBD including inhibiting IL-1 release by intestinal epithelial cells, preventing lymphocyte recruitment, inhibiting platelet activating factor (PAF) and manipulating eicosanoid metabolism (Greenfield *et al.*, 1993).

There are, however, important clinical, immunological and hence therapeutic differences between Crohn's Disease and Ulcerative Colitis which are proving ever more important as our understanding of these conditions improves. These are outlined below and in Table 1.1.

Feature	Crohn's Disease	Ulcerative Colitis
Distribution	Entire length of gut	Limited to Colon
Thickness of inflammation	Full thickness	Limited to mucosa and submucosa
Genetic associations	Specific to CD	Specific to UC
Pattern of immune response	Th1 pattern	Modified Th2
Effect of smoking	Associated	Protective
Diversion of faecal stream	Relieves symptoms	Exacerbates symptoms
Response to antibiotics	Partial	None
Response to Diet	Partial	None
Response to anti-TNFα therapy	Partial	None

Table 1.1: ***Differences between Crohn's Disease and Ulcerative Colitis***

1.1.2.1 Crohn's Disease

Crohn's Disease can affect any part of the entire length of the gastrointestinal tract, either in isolation or in combination. Furthermore the entire thickness of the bowel wall can be involved with an inflammatory reaction on the serosal surface, and the inflammatory process is characterised by the presence of granulomas. However despite this potential for variety there are classic patterns of disease such as:

- Ileo-caecal disease
- Peri-anal disease
- Stricturing and fistulising disease
- Crohn's colitis

The reason that such clinical manifestations may be important, particularly in the absence of a unifying aetiological process, is that Crohn's Disease may represent an agglomeration of related but different diseases with subtle differences in their

genetic, immune and environmental profiles. There is evidence supporting this hypothesis with the finding that lymphocytes expressing the C-C chemokine receptor 9, as well as secretion of the chemokine TECK, differentiate small bowel from colonic Crohn's disease (Papadakis *et al.*, 2001). Furthermore, recent genetic evidence demonstrates a link between the apoptosis regulator, and NFκB activator, NOD2 and susceptibility to Crohn's disease (Hugot *et al.*, 2001).

Generally Crohn's Disease displays a classic Th1 type pattern of immune response typified by increased IFNγ, TNFα, IL-1 and IL-12 (Shanahan, 2001; Fiocchi, 1998). It is perhaps because it fits into this immunological pattern of response that there have been more recent advances in immunotherapies for Crohn's Disease than there have for Ulcerative Colitis. The most striking of these has been using TNFα as a therapeutic target for the treatment of Crohn's Disease. Using a variety of techniques to block the action of TNFα in Crohn's Disease has resulted in dramatic therapeutic benefits. The most studied of these is a chimeric monoclonal antibody against TNFα (Infliximab or Remicaide®) which is widely used and, interestingly, is particularly effective in perianal and fistulising Crohn's Disease implying that TNFα may be particularly important in these conditions (Present *et al.*, 1999).

Following this lead there is now extensive research into immunotherapies in IBD (Shanahan, 2001), and Crohn's Disease in particular, with many already at the trial stage as summarised in the table (1.2) below.

Potential Method of Action	Example	Reference
1. Antibodies to Pro-Inflammatory Molecules	• *Anti TNFα Antibody**** • *Anti Integrin Antibody***	(Targan *et al.*, 1997) (Gordon *et al.*, 2001)
2. Human Recombinant forms of Anti-Inflammatory Molecules	• *Interleukin-10*** • *Interleukin-11***	(Van Deventer *et al.*, 1997) (Bank *et al.*, 1997)
3. Antagonists to Receptors for Pro-Inflammatory Molecules	• *Interleukin-1 receptor antagonist** • *TNFα receptor antagonist (Etanercept)***	(Cominelli *et al.*, 1990) (D'Haens *et al.*, 2000)
4. Antisense RNA to block translation of Pro-Inflammatory Molecules	• *ICAM Antisense*** • *Nuclear Factor κB Antisense**	(Yacyshyn *et al.*, 1998) (Neurath *et al.*, 1996)
5. Antagonists for Enzymes making Pro-Inflammatory Mediators	• *Lipoxygenase Antagonists*** • *TNFα converting enzyme (TACE) antagonists**	(Hawkey *et al.*, 1997) (Dekkers *et al.*, 1999)
6. Specific Antagonists of Pro-Inflammatory Molecules	• *Platelet Activating Factor Antagonist***	(Stack *et al.*, 1998)

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** Clinical Trials

* Animal Work only

Table 1.2: ***Different molecular targets for therapies in IBD***

1.1.2.2 Ulcerative Colitis

From the point of view of the gastrointestinal tract, Ulcerative Colitis only affects the colon and always works proximally from the rectum in a continuous fashion. The

chronic inflammatory process only involves the mucosa and submucosa sparing the muscularis layers of the bowel. Although often referred to as a disease with a Th2 type immune response, in contrast to Crohn's Disease, this is an oversimplification and cytokine patterns in Ulcerative Colitis are less clear (Shanahan, 2001). In particular it appears that the type of immune response alters in the acute and chronic phases of disease. In established disease there are features of a modified Th2 response with a cytokine profile involving IL-5 and IL-10. This complexity in classifying the immune response reflects the generally poor results of immune based therapies in this condition. Indeed, the breakthroughs made with Crohn's Disease have not worked in Ulcerative Colitis, exemplifying how these conditions are diverging as therapies progress.

1.1.3 Colorectal Carcinoma

The gastrointestinal tract is the commonest site of internal malignancies, with cancers most likely to develop in the colon. Colorectal cancer, like IBD, is predominantly a disease of the western world where it is the third most common cancer and the second most common cause of cancer related death. The World Health Organisation estimated that in 1996 there were 875,000 new cases of colorectal cancer worldwide and that there would 495,000 deaths attributable to the condition. Not only is this an important condition from the point of its high incidence but also because its genetic component has revealed much information on intestinal tumourigenesis.

It is only the minority of colorectal carcinomas which are associated with an inherited genetic defect (Chung, 2000). The most important of these are hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome, which accounts for between 5-10% of cases, and the autosomal dominant condition familial adenomatous polyposis (FAP) accounting for approximately 1%. The majority of the remaining cases are termed sporadic.

FAP is associated with disruption of the tumour suppressor gene *APC* which was cloned in 1991 (Groden *et al.*, 1991; Nishisho *et al.*, 1991). The APC gene product is a large protein with multiple functional domains that mediate both oligomeristaion and binding to many intracellular proteins including β-catenin, γ-catenin, glycogen synthase kinase (GSK)-3β, axin and tubulin. The most progress in understanding its function comes from studies of its interaction with GSK-3β and β-catenin, each an essential component of the Wingless/Wnt signalling pathway which was first characterised in *Drosophila.* In the normal physiological state there is a normal APC gene product and no Wnt signal. In these conditions, GSK-3β associated with APC phosphorylates β-catenin resulting in its degradation. In a tumourigenic state there is either a Wnt signal, or a mutant APC or β-catenin. The result of this is that β-catenin is not degraded, but accumulates in the cytoplasm and nucleus where it binds to T-cell factor (TCF) transcription factors resulting in the up-regulation of oncogenes such as c-Myc, cyclin D1 and, putatively, PPARδ.

This work on APC combined with work on two other tumour suppressor gene pathways, K-ras and p53 which are mutated in approximately 50% of colorectal carcinomas, resulted in the proposal of a model of sporadic intestinal tumourigenesis

by Fearon and Vogelstein. This model has proved to be very durable and able to accommodate the many new discoveries since its proposal in 1990 (Fearon and Vogelstein, 1990). It proposes a series of genetic mutations which allow the progression through the adenoma-carcinoma sequence. This progression occurs over a long time period of approximately a decade and is illustrated in Figure 1.3.

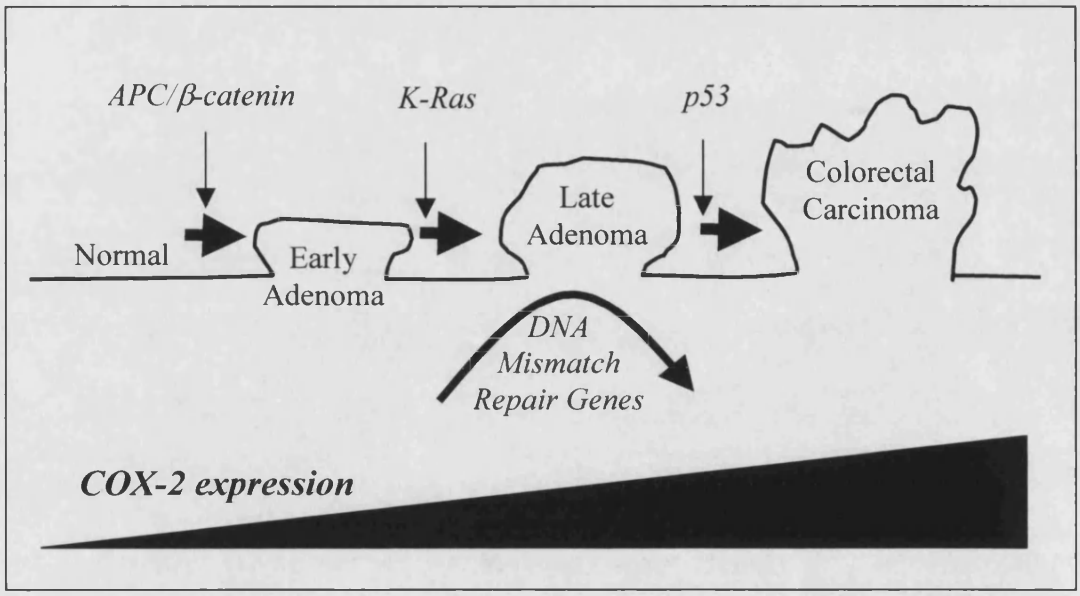


Figure 1.3: ***Model of colorectal carcinoma progression***

The clinical phenotype of HNPCC, and newly discovered information on the associated genotype, has added significant information to this model. HNPCC presents at a younger age than sporadic cases, predominantly affects the right colon, follows an autosomal dominant pattern, and appears to progress through the adenoma-carcinoma sequence much faster than the decade taken for sporadic cases. This phenotype is now explained by the discovery of the basic genetic defect which is an inactivating mutation in one of the DNA mismatch repair genes (*MSH2, MLH1, PMS2, PMS1, MSH6*). This inactivation may be due to a germline mutation, or

epigenetic phenomena best illustrated by hypermethylation in the promoter causing inactivation, or a combination of both in a classic two hit model of tumour suppressor gene inactivation.

The resulting disruption of the ability to replicate DNA accurately is most noticeable in the short tandem repeats, known as microsatellites, and results in a high spontaneous rate of mutation. Subsequent microsatellite instability (MSI) in the associated tumours is also called replication error positivity (or RER+). An important gene which is particularly susceptible to mismatch repair mutations is that for the type II transforming growth factor (TGF)-β receptor. TGF-β is a potent inhibitor of colonic epithelial cell growth and an acquired disruption in its receptor has been shown to contribute to HNPCC.

1.1.4 Inflammatory Bowel Disease and colorectal carcinoma

It has become apparent that the presence of chronic colonic inflammation predisposes to colorectal carcinoma (Morson, 1985; Bansal and Sonnenberg, 1996). This increased risk is dependent on the extent of colonic involvement and the length of time since the onset of disease. As the predisoposition to colon cancer is highest in those with colonic inflammation involving the whole colon for at least eight years, it is unsurprising that the association between IBD and colorectal carcinoma is best seen in ulcerative colitis. However, chronic Crohn's colitis also carries an increased risk of malignant transformation although the association is less marked (Bansal and Sonnenberg, 1996).

1.2 Cyclo-oxygenase

Many immunologically active mediators have been proposed as playing a pathological role in IBD and colorectal carcinoma, and some of these have been targets for novel therapeutic strategies (see Table 1.2). This thesis will concentrate on the action of the enzyme Cyclo-oxygenase and its dependent products.

1.2.1 History

Cyclo-oxygenase is the name given to enzymes capable of converting arachidonic acid to prostanoids. Although the first cyclo-oxygenase enzyme (COX), also known as prostaglandin synthase (PGS) or prostaglandin G/H synthase (PGHS), was isolated in 1976 (Hemler *et al.*, 1976), this was late in the history of prostaglandin production because the effects of inhibiting COX were described by Hippocrates. Indeed the whole field of cyclo-oxygenase research is intertwined with the existence of their inhibitors.

The use of powder from the dried bark of the willow tree (*Cortex salicis*) to treat fever in fifty patients was described in 1763 (Stone, 1763) with the active ingredient, salicylic acid, being commercially synthesised in 1859. However, although widely used as an anti-pyretic and analgesic, side effects of gastric irritation were troublesome. Felix Hoffman, an employee of Friedrich Bayer, tackled this problem in 1897 by synthesising a stable form of acetyl salicylic acid. He named this A-Spirin, the A representing the acetylation step and Spirin from *Spirea ulmaria* or

meadowsweet, a plant rich in salicylates. Bayer patented Aspirin and started marketing it in 1899 with vast success, hence starting the modern pharmaceutical industry.

Aspirin was the first of a group of drugs, collectively known as non-steroidal anti-inflammatory drugs (NSAIDs), whose use became widespread. However, their mechanism of action remained unknown until 1971 when Vane described their inhibition of prostaglandin synthesis for which he won the Nobel Prize for Medicine in 1982 (Vane, 1971). This was followed by the purification of the first cyclo-oxygenase enzyme in 1976 (Hemler *et al.*, 1976) allowing the subsequent characterisation of the catalytic mechanisms and methods of NSAID induced inhibition.

At this point it was believed that there was only one COX enzyme which was constitutive in nature and that the rate limiting step for prostaglandin synthesis was the release of arachidonic acid substrate by the activation of phospholipases. This was shown not to be correct with the isolation of cDNA for the one known COX enzyme (PGS EC 1.14.99.1) (Yokoyama and Tanabe, 1989), and the concomitant demonstration that there was an inducible component to COX activity that was independent of both phospholipase induction and any increase in PGS EC 1.14.99.1 message (Masferrer *et al.*, 1990). The subsequent search for an inducible COX gene resulted in the isolation of an inducible cDNA encoding a novel COX enzyme by two groups simultaneously (Kujubu *et al.*, 1991; Xie *et al.*, 1991). These two isoforms of COX, the initially discovered classically constitutive component COX-1,

and the second inducible component COX-2, remain the only COX enzymes currently identified.

1.2.2 Arachidonic Acid metabolism

COX enzymes catalyse the conversion of arachidonic acid to prostaglandins – firstly by inserting two oxygen molecules to yield PGG_2 (cyclo-oxygenase activity) and then reducing this intermediate to give PGH_2 (peroxidase activity). PGH_2 is subsequently converted to a variety of eicosanoids, arachidonate derivatives based around a 20 carbon structure (eicosa is Greek for twenty), that include the prostaglandins and thromboxane A_2. Which eicosanoids are produced is dependent on the environment, as well as the enzymatic machinery, of particular cells (Figure 1.4).

To put the COX enzymes in the context of arachidonic acid metabolism as a whole, one needs to start at the level of stimulus initiated arachidonic acid liberation from phospholipid, a reaction catalysed by phospholipases A, C and D. The phospholipase A_2 (PLA_2) family of enzymes are the most important with respect to prostaglandin production, and can be split into secretory or $sPLA_2$ (low MWt 14-18kDa), and cytoplasmic or $cPLA_2$ (high MWt 80-110 kDa). They represent a potential point of regulation of eicosanoid production and respond to agonist stimulation. Indeed they have been shown to be up-regulated in both Crohn's disease and Ulcerative Colitis (Minami *et al.*, 1994; Haapamaki *et al.*, 1999). Furthermore, there is evidence for some form of coupling between phospholipases and cyclo-oxygenases, although the mechanism for this is yet to be elucidated. It has

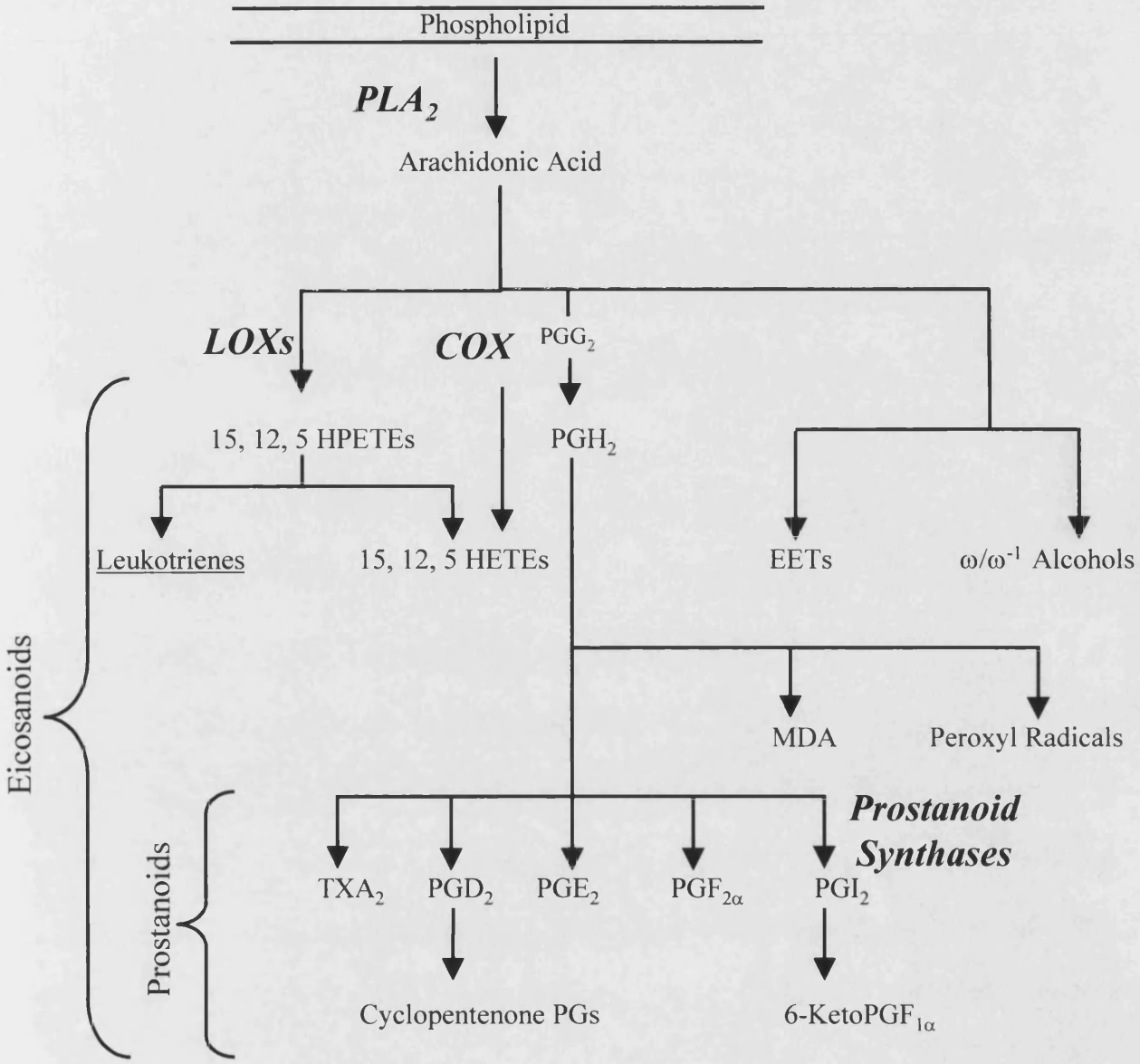


Figure 1.4: ***Pathways of Arachidonic Acid metabolism***
Schematic representation of the pathways involved in arachidonic acid metabolism and generation of eicosanoids. PLA_2 phospholipase A_2; LOX lipoxygenase; COX cyclooxygenase; PG prostaglandin; HPETE hydroperoxyeicosatetraenoic acid; HETE hydroxyeicosatetraenoic acid; EET epoxyeicosatetraenoic acid; MDA malondialdehyde

been hypothesised that different phospholipases selectively provide arachidonic acid to COX-1 or COX-2. Such a mechanism would probably depend on coincidental enzyme expression rather than a unique metabolic interaction (Herschman, 1996; Reddy and Herschman, 1997; Ueno *et al.*, 2001).

Having generated free arachidonic acid, it can be metabolised by one of three pathways:

- Lipoxygenases
- Cytochrome p450
- Cyclo-oxygenases

Lipoxygenases insert an oxygen molecule into the twenty carbon ring of arachidonic acid and are numbered after the target carbon atom with 5-, 12-, and 15-lipoxygenase being the major enzymes relevant to gastrointestinal function. The ultimate products of lipoxygenase (LOX) activity are the leukotrienes, lipoxins and hydroxy fatty acids such as the hydroperoxyeicosatetraenoic (HPETEs) and hydroxyeicosatetraenoic (HETEs) acids. Of particular mechanistic interest is the finding that for 5-LOX, which catalyses leukotriene biosynthesis, there is an associated 5-LOX activating protein (FLAP) which is essential for functional 5-LOX activity. This 18kDa protein associates with 5-LOX at the nuclear membrane and acts as an arachidonic acid transfer protein which presents the substrate to the enzyme. Such a mechanism appears to be unique, although it has been hypothesised to exist for COX in order to explain selective substrate availability between COX isoforms (Fitzpatrick and Soberman, 2001).

The cytochrome p450 pathway of arachidonic acid metabolism represents a reaction where a single oxygen atom is inserted resulting in epoxy and (ω/ω^{-1}) derivatives. Less is known about the functional importance of this pathway. Finally, and as an alternative to the above three pathways of arachidonic acid metabolism, arachidonic acid can be converted non-enzymatically to isoprostanes via free radical peroxidation, or be re-esterified to give phospholipid. These pathways are represented in Figure 1.4

1.2.3 COX-1

COX-1 is encoded by a 24kb gene yielding a 2.8kb mRNA which in turn yields a 71kDa protein. The gene does not have a TATA box in its promoter and contains multiple start sites of transcription. The only proven *cis*-regulatory elements in the promoter are two SP1 sites at positions –111/-105 and –610/-604. Deletion of one or both of these sites results in a reduction of basal transcription of 50% or 75% respectively in human umbilical vein endothelial cells (Xu *et al.*, 1997). It is classically regarded as being constitutive but is preferentially expressed at high levels in selected cells and tissues, including endothelial cells, platelets, renal collecting tubules and seminal vesicles demonstrating that it is developmentally regulated. In addition it is possible to induce modest increases in COX-1 mRNA when inducing a monocytic cell line, THP-1, to differentiate into a more macrophage-like cell line using TPA, again indicating a developmental regulation (Smith *et al.*, 1993). In the gastrointestinal tract it has been shown that γ-irradiation of crypt epithelial cells induces COX-1 (Cohn *et al.*, 1997) but there are no other examples of COX-1 inducibility in this system.

1.2.4 COX-2

COX-2 is encoded by an 8.8kb gene which is transcribed into a 4kb mRNA which in turn produces a protein of 65kDa to 74kDa depending on its extent of glycosylation (Otto *et al.*, 1993). This COX-2 protein demonstrates a 61% homology to COX-1 in humans with both enzymes displaying similar kinetics (Vane and Botting, 1996). Its main structural feature distinguishing it from COX-1 is an 18 amino acid sequence at the carboxy terminus whereas the COX-1 protein has a 17 amino acid sequence at the amino terminus. These features act as specific epitopes allowing the generation of specific antibodies to the two isoforms.

The gene structure for COX-2 is markedly different than that for COX-1. COX-2 is an immediate early (IE) gene (or primary response gene) which can be expressed in the absence of new protein synthesis. As with all IE genes it is relatively small but has functionally important regulatory regions at both the 5′ and 3′ ends of the gene. These allow regulation at the transcriptional and post-transcriptional levels. Indeed, in studies of cytokine induction of COX-2 in macrophages where cytokines caused a 40 fold increase in COX-2 mRNA, there was only a 10-40% increase in transcription with alteration of mRNA stability causing the majority of the mRNA increase (Huang *et al.*, 2000).

The promoter region at the 5′ end of the COX-2 gene has been shown to have numerous putative elements regulating transcription although only five have been proven to regulate COX-2 transcription (See Figure 1.5):

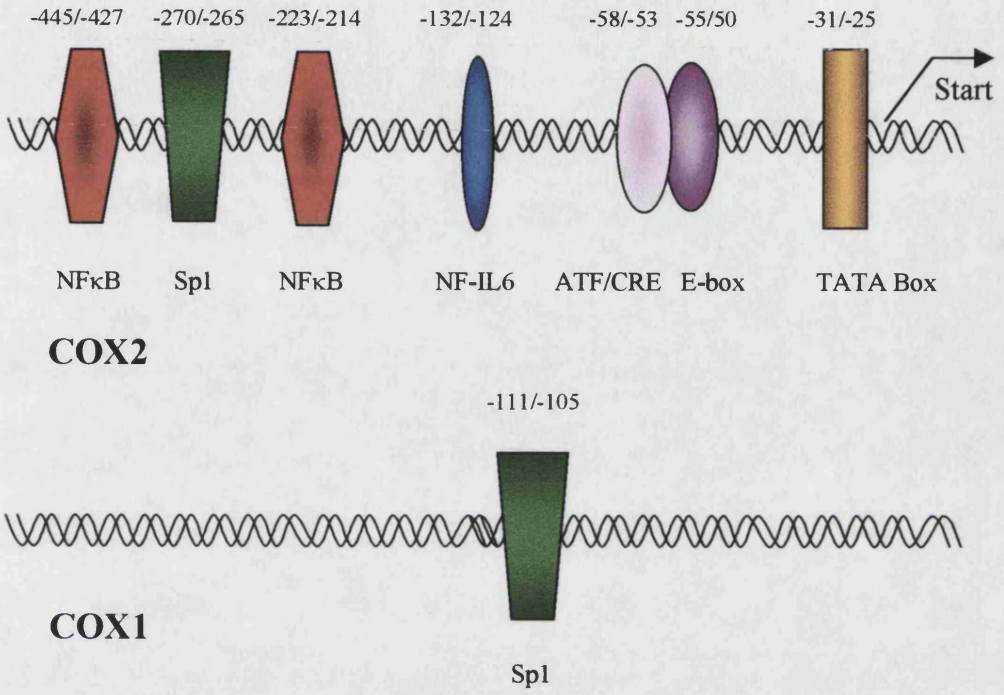


Figure 1.5: ***Regulatory Elements in the COX1 and COX2 Promoter***
A schematic representation of the COX1 and COX2 promoters showing the binding sites for transcription factors in the 500bp prior to the start point of transcription. All elements shown have been proven to have a regulatory role.

- Nuclear Factor κ B (NFκB)
- SP1
- Nuclear Factor IL-6 (NF-IL6)
- ATF/cAMP response Element (CRE)
- E-Box

However deletion studies of promoter constructs have shown that regions up to 2300 bases 5′ of the TATA box do regulate TGFβ induced COX-2 transcription (Yang *et al.*, 1997). Such studies have shown varied results in different cell lines (Reddy *et al.*, 2000) and there appears to be cell type specificity in the promoter components which are essential to transcription.

At the 3′ end of the COX-2 gene is an untranslated region (3′-UTR) which also plays a major role in COX-2 expression (Figure 1.6). This region contains multiple copies of adenylate and uridylate rich elements (AREs), composed of the sequence 5′-AUUA-3′, which allow regulation of both mRNA stability and translational rate (Dixon *et al.*, 2000). Inhibition of *de novo* protein synthesis with cycloheximide causes an up-regulation of COX-2 mRNA in numerous systems (Huang *et al.*, 2000; Newton *et al.*, 1997). It seems that, although transcription of COX-2 does not require protein synthesis, when it is induced proteins are made which will subsequently down regulate it at the level of mRNA stability and translational rate and these function by binding to the 3′-UTR. An example of such a protein is tristetraprolin (Taylor *et al.*, 1996; Carballo *et al.*, 2000).

The above regulatory elements indicate that COX-2 is an inducible gene which is highly regulated at many different points (Figure 1.7). Indeed, COX-2 can be

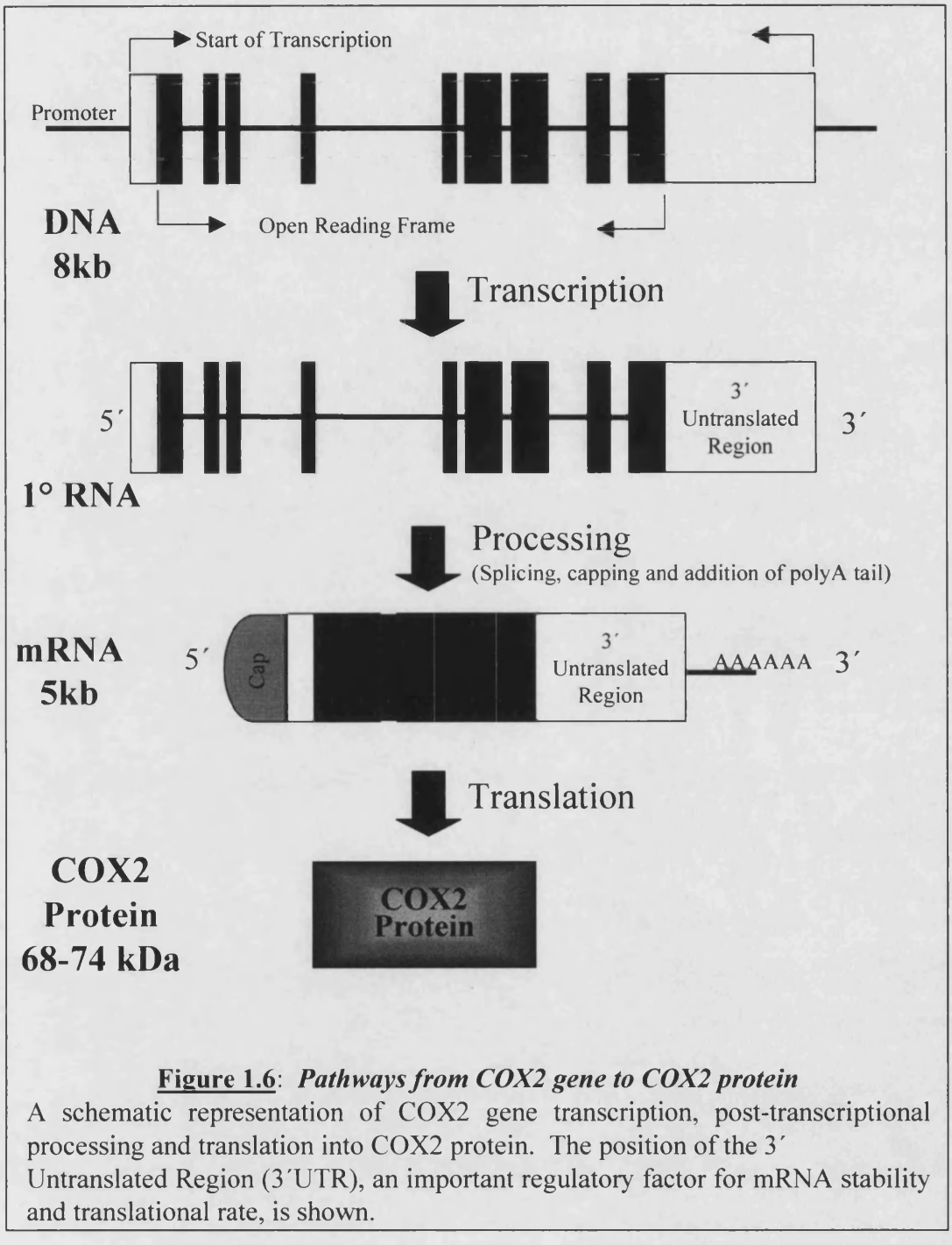


Figure 1.6: ***Pathways from COX2 gene to COX2 protein***
A schematic representation of COX2 gene transcription, post-transcriptional processing and translation into COX2 protein. The position of the 3′ Untranslated Region (3′UTR), an important regulatory factor for mRNA stability and translational rate, is shown.

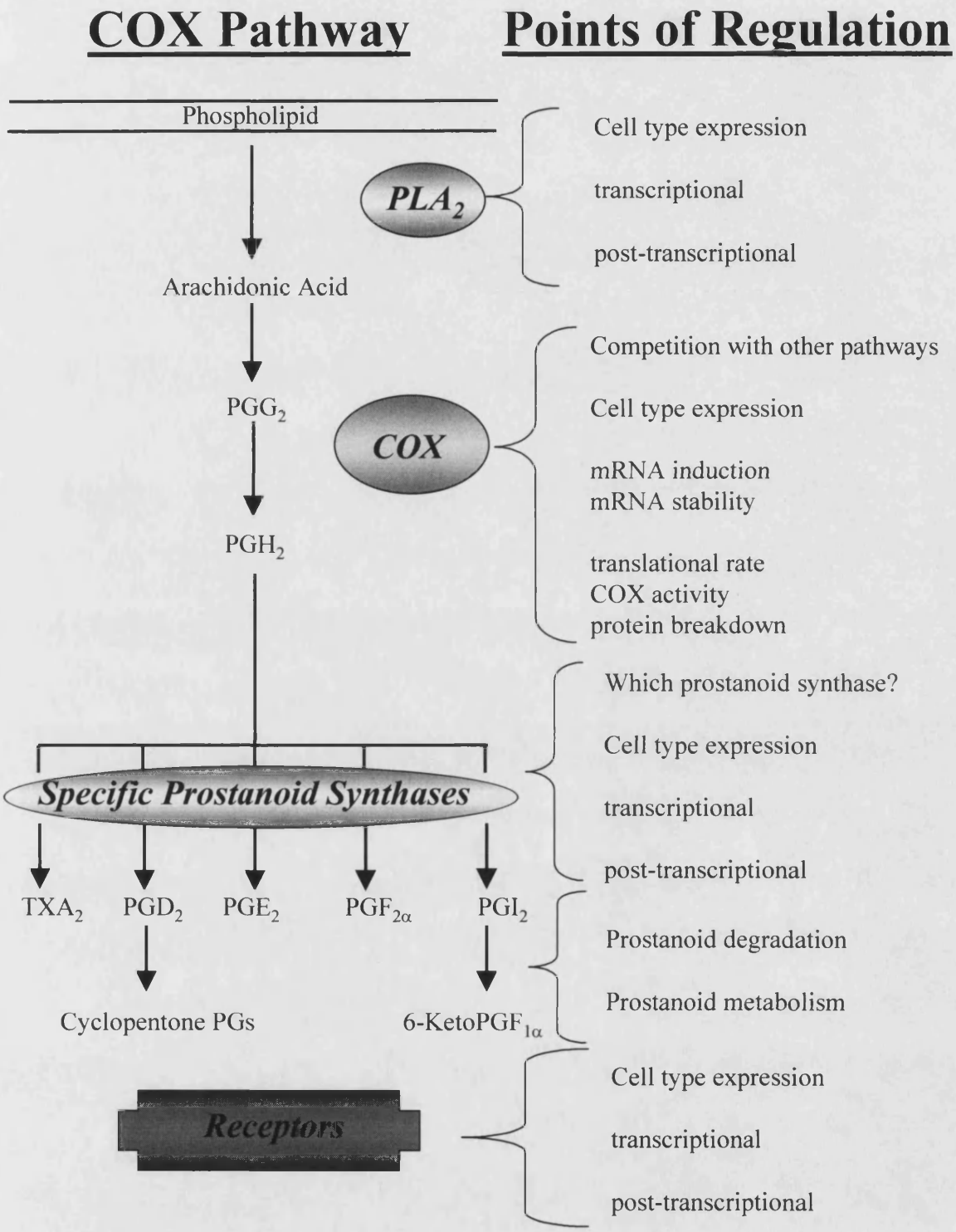


Figure 1.7: ***The COX pathway and its potential points of regulation***
Schematic representation of the COX pathway including upstream and downstream components. Potential points of regulation are shown to the right of the figure.

induced by a number of stimuli in a variety of systems. These include pro-inflammatory cytokines, bacterial products and bacterial invasion, oncogene expression, growth factors, serum, high osmolarity and ultraviolet radiation (Smith *et al.*, 2000). Correspondingly, COX-2 has been shown to play an important role in physiological and pathophysiological processes throughout the body, and in inflammatory processes and cancer development particularly (DuBois *et al.*, 1998).

1.2.5 Cyclo-oxygenase Products and their Receptors

COX activity is the committed step to prostaglandin synthesis. However, COX is also capable of synthesising malondialdehye or peroxyl radicals, as well as 15-R-hydroxyeicosatetraenoic acid (HETE) (Shiff and Rigas, 2001) which is particularly important in the context of Aspirin use (Gronert *et al.*, 1998). This 15-(R)-HETE compound enters the pathways downstream of lipoxygenase and acts as a precursor of 15-epi-lipoxin A_4 biosynthesis which in turn inhibits IL-8 release.

From the point of view of prostaglandins, which prostaglandin is synthesised as a result of COX activity depends on the specific downstream prostanoid synthase. There is also evidence that these prostanoid synthases can couple with either COX-1 or COX-2. Such coupling of specific cyclo-oxygenase isoform and prostanoid synthase can either be due to co-ordinate induction, or potentially by direct interaction although there is no evidence of the latter (Smith *et al.*, 2000). This coupling may explain the inverse regulation of PGD_2 and PGE_2 by TNFα activation of murine macrophages (Fournier *et al.*, 1997). As well as producing thromboxane A_2 by a specific synthase, the other prostaglandins that COX can potentially produce

include PGE_2 – the major prostaglandin product in the gastrointestinal tract (Boughton-Smith *et al.*, 1983), PGI_2 or prostacyclin, $PGF_{2\alpha}$ and finally PGD_2. The latter prostaglandin is metabolised into members of the cyclopentenone prostaglandin family (Willoughby *et al.*, 2000).

Which prostaglandin is synthesised by specific cell types is dependent on the prostanoid synthases expressed. As examples of this: platelets mainly express TXA_2 and vascular endothelial cells mainly PGI_2; mast cell synthesise PGD_2 but not PGE_2. Despite this cell specialisation it has been demonstrated that the gastrointestinal tract can generate the entire range of eicosanoids (Bennett *et al.*, 1981). Once synthesised, prostaglandins cannot be stored and act as local paracrine, autocrine or intracrine mediators. They are involved in a multiplicity of physiological responses and mediate the effects of COX expression.

Two classes of prostaglandin receptors exist to transduce signals upon ligand binding. Firstly, the G protein coupled cytoplasmic membrane class of receptor and secondly the peroxisome proliferator activated receptor (PPAR) class of nuclear receptors (Versteeg *et al.*, 1999). All these types of eicosanoid receptors display considerable cell type specific expression allowing another point of extensive regulation of the physiological response to prostaglandins. These receptors, and the effects of ligand binding, are summarised in Table 1.3. PPAR receptors will be considered later in the introduction with transcription factors.

Eicosanoid	Receptor	Effect
PGE_2	EP1	↑IP3/DAG
	EP2	↑cAMP
	EP3	↓Gi/cAMP
	EP4	↑cAMP
$PGF_{2\alpha}$	FP	↑IP3/DAG
TXA_2	TP	↑IP3/DAG
PGI_2	IP	↑cAMP
	PPARδ	
PGD_2	DP	↑cAMP
PGJ_2	***PPARγ***	

Table 1.3: ***The eicosanoid receptors and the effect of individual ligand binding***

1.2.6 Cyclo-oxygenases and the gastrointestinal tract

1.2.6.1 Normal epithelial function

COX-1 is expressed throughout the normal gut as shown by isolation of mRNA (O'Neill and Ford-Hutchinson, 1993), protein (Kargman *et al.*, 1996), and immunohistochemistry (Singer *et al.*, 1998). It is expressed in the lower crypt epithelial cells corresponding to areas of proliferation in the small and large intestine (Cohn *et al.*, 1997; Singer *et al.*, 1998). Such a position implies a function in promoting normal, homeostatic proliferation and this is supported in studies of

regeneration from irradiation induced injury. COX-1 knockout mice (*COX-1-/-*) have no gastrointestinal phenotype in the unstressed state although do show impaired response to injury (Cohn *et al.*, 1997).

The action of prostaglandins in the normal functioning of the gastrointestinal tract is controversial. There is pharmacological evidence of a role for prostaglandins in physiological intestinal secretion and motility (Eberhart and DuBois, 1995). However, COX-1 (*COX-1-/-*) and COX-2 (*COX-2-/-*) deficient knockout mice have no appreciable intestinal phenotype apart from a mild predisposition to peritonitis in *COX-2-/-* mice (Morteau *et al.*, 2000). This is despite a total inhibition of basal PGE_2 production in *COX-1-/-* mice and either implies that compensatory mechanisms have developed in the mice or that COX activity is not needed for the normal gastrointestinal function in the unstressed gut.

COX-2 is not expressed in the normal gastrointestinal tract. However it is abundantly expressed in both cancers, and inflammatory processes of the gut.

1.2.6.2 NSAIDs and the upper gastrointestinal tract

Before concentrating on the colon it is important to briefly consider the actions of NSAIDs on the stomach. The ability of salicylates to cause gastric irritation was realised prior to the production of Aspirin at the end of the 19th century. Since then it has been widely appreciated that NSAIDs cause peptic ulceration. This, combined with the discovery of the two isoforms of COX, resulted in the paradigm that COX-1 was crucial to gastric mucosal protection and COX-2 was related to inflammation. As it was only at the very end of the 20th century that COX selective inhibitors

became available, it was assumed that the injurious effects of classical NSAIDs to the stomach were due to COX-1 inhibition and the beneficial effects as analgesics was due to their inhibition of COX-2.

However, although the above theory provided a good working model, it could not explain why *COX-1-/-* mice have no gastric ulceration (Morteau *et al.*, 2000). Furthermore, it could not explain findings from experiments using both highly selective COX-1 and COX-2 inhibitors. A more complex interaction is emerging with evidence that COX-2 is required for ulcer healing (Mizuno *et al.*, 1997), an important consideration for those developing ulceration on a COX-2 selective inhibitor. Also, in rats, specific pharmacological COX-1 inhibition does not cause gastric ulceration although a combination of specific COX-1 and COX-2 inhibitors was injurious (Wallace *et al.*, 2000). In summary, the classical view of only COX-1 contributing to mucosal defence is no longer tenable, although specific COX-2 inhibition (or COX-1 sparing NSAIDs) is less injurious to the stomach than standard NSAIDs.

1.2.6.3 COX and colorectal carcinoma

The links between COX activity and colorectal carcinoma are strong and have been the focus of intense scientific research. Prior to the discovery of COX, human colorectal carcinomas had been demonstrated to produce more PGE_2 than the surrounding normal mucosa (Bennett and Del Tacca, 1975). This was followed by the clinical observation that patients with familial adenomatous polyposis (FAP) had a regression of their polyps when placed on the NSAID sulindac (Waddell and Loughry, 1983). Epidemiological studies subsequently showed that long term

NSAID use, and aspirin in particular, caused a significant reduction in mortality due to colorectal carcinoma (Giovannucci *et al.*, 1994; Giovannucci *et al.*, 1995). Having demonstrated that NSAIDs prevent colorectal carcinoma, the role of the isoforms of COX in the mechanism of this needed to be investigated.

With the discovery of the inducible isoform of COX it became apparent that, although COX-1 expression was unaltered in colorectal carcinoma, the expression of COX-2 was significantly increased (Eberhart *et al.*, 1994). COX-2 has been shown to be expressed in 80-90% of colorectal carcinomas and 40-50% of premalignant adenomas. Furthermore, its specific inhibition causes a reduction in polyps in humans with FAP (Steinbach *et al.*, 2000). In animal models, *APCΔ716(+/-)* mice, which develop hundreds of intestinal tumours mimicking FAP, develop far fewer tumours when crossed with *COX-2-/-* mice. This is further supported by animal models based on the injection of tumour cell lines into host animals. Injection of $COX\text{-}2^{+}$ and $COX\text{-}2^{-}$ xenografts into animals showed that the COX-2 expressing tumours grew far better, and that this was abrogated by COX-2 inhibition (Tsujii *et al.*, 1998). This same study also showed that COX-1 had a role in regulating pro-angiogenic factors necessary for tumour development. This study was followed by performing a similar experimental protocol in reverse with COX-2 expressing tumour cell lines being injected into *COX-1-/-* or *COX-2-/-* mice. The tumours grew far slower in *COX-2-/-* mice compared to the *COX-1-/-* and wild type mice showing that host COX-2 as well as tumour COX-2 play a crucial role (Williams *et al.*, 2000).

Thus COX activity generally is crucial to colorectal cancer development, with the particular importance of COX-2 probably being secondary to its inducibility and

hence generation of a greater quantity of eicosanoid products. This effect of COX-2 has been referred to as a landscaping or a field effect within the Vogelstein and Kinzler paradigm (Figure 1.3) (Kinzler and Vogelstein, 1998). Indeed, it may be the mechanism by which chronic IBD predisposes to colorectal carcinoma, another landscaping effect (Agoff *et al.*, 2000; Kinzler and Vogelstein, 1998; Bansal and Sonnenberg, 1996). The expression of COX in epithelial cells, stromal cells and endothelial cells has been show to make a contribution to tumourigenesis in different models but the relative contributions of each cell type remain uncertain. Also, which COX products are most important in cancer development, and their mechanisms of action, needs further investigation.

As well as the undisputed importance of COX inhibition in the chemopreventive actions of NSAIDs, it is also apparent that NSAIDs can exert a number of COX independent effects. This is most clearly demonstrated by the fact that metabolites of certain NSAIDs, such as sulindac sulfone, which have no COX inhibiting properties, still cause decreased proliferation and increased apoptosis in models of colon cancer (Elder *et al.*, 1996; Piazza *et al.*, 1997). Secondly colonic cell lines, such as HCT-15 which completely lack COX activity and the ability to synthesise prostaglandins, still respond to NSAIDs in a similar manner to COX expressing cell lines (Hanif *et al.*, 1996). Thus there is undoubtedly a COX independent component to the action of NSAIDs which is likely to be dose dependent and which may be seen predominantly at toxic doses (Williams *et al.*, 1997).

1.2.6.4 COX and Inflammatory Bowel Disease

The eicosanoid products of cyclo-oxygenase were known to be both produced by the colon (Boughton-Smith *et al.*, 1983), and raised in IBD, prior to the discovery of an inducible isoform of COX (Donowitz, 1985; Lauritsen *et al.*, 1988). Furthermore, these eicosanoid products were produced at times of active disease and returned to basal levels during remissions implying an inducible component (Sharon *et al.*, 1978). Hence, with the discovery of COX-2, it was hypothesised that it would be induced in IBD. This has now been demonstrated with the discovery of increased COX-2 mRNA in IBD which was shown to reflect disease activity (Hendel and Nielsen, 1997). This was followed by isolation of protein, along with immunohistochemistry, showing increased COX-2 expression in ulcerative colitis as well as in Crohn's colitis and ileitis (Singer *et al.*, 1998). The immunohistochemical staining showed that, although COX-2 was expressed in both epithelial cells and lamina propria mononuclear cells, the vast majority of the protein was in the epithelial cells.

Having confirmed the induction of COX-2 in IBD, the functional role of this expression needs to be assessed. It has been known for some time that NSAIDs cause exacerbation of IBD although it was not known whether this was a COX specific action (Kaufmann and Taubin, 1987). Three lines of evidence imply that this exacerbation of IBD is due to COX-2 inhibition.

1. The specific COX-2 inhibitor, L745,337, exacerbates a rat model of trinitro benzene sulphonic acid (TNBS) induced colitis resulting in higher rates of perforation and death (Reuter *et al.*, 1996).

2. Analogously, colitis induced in mice by oral Dextran Sodium Sulphate (DSS) administration is more aggressive and more lethal in *COX-2-/-* mice (Morteau *et al.*, 2000). *COX-1-/-* mice were also more susceptible to induced colitis, although not as marked as the *COX-2-/-* animals, and this suggests a protective role for both COX-1 and COX-2 expression in the context of IBD.

3. Finally COX dependent products are protective in two animal models of colitis. Firstly, intra-rectal administration of PGE_2 (100μg) in rats from one week after exposure to (DSS) resulted in histological amelioration of the colitis compared to controls (Sasaki *et al.*, 2000). Also, myeloperoxidase activity around the lesions was less and there was decreased expression of the chemokines GRO and CINC-1 in the treated group. Secondly, using TNBS to induce colitis in rats, PGD_2 was shown to be increased after the induction of colitis and that its action was to reverse the inflammatory process. Again colitis could be ameliorated by administration of PGD_2 (Ajuebor *et al.*, 2000).

Thus it seems that COX expression, and the induced COX-2 expression in particular, is beneficial in IBD and that this is dependent on the COX mediated production of eicosanoids such as PGE_2 (McCartney *et al.*, 1999).

1.3 Cytokines

1.3.1 Tumour Necrosis Factor α

The tumour necrosis factor protein was isolated in 1985 and named after its ability to kill tumour cells. It was subsequently found to refer to two closely related cytokines, TNFα and TNFβ (or lymphotoxin-α) which share receptors and play a part in a wide variety of human diseases processes. TNFα is a member of a larger family of related ligands and is an immediate early gene which shares many of the characterisitic of the COX-2 gene: specifically-

- Multiple response elements in its promoter. These include binding sites for NFκB, AP-1, AP-2, SP1 and CRE and there is evidence of combinatorial activation as with COX-2.
- The presence of AREs in the 3′-UTR allowing post-transcriptional regulation via control of mRNA stability and translational rate.
- Expression in monocytes and macrophages, as well as B and T lymphocytes and also intestinal epithelial cells (Jung *et al.*, 1995).
- Induced by a large number of diverse stimuli such as cytokines, growth factors, bacterial products and irradiation.

TNFα is first synthesised as a transmembrane (26kDa) precursor which can be biologically active in a juxtacrine manner. This precursor can then be cleaved by TNFα converting enzyme (TACE) into the 17kDa secreted form of TNFα which combine into homotrimers to bind to and activate its receptors.

There are two distinct TNFα receptors which are also part of a larger family of related receptors. These receptors, TNFR1 (p55 or CD120a) and TNFR2 (p75 or CD120b), are expressed simultaneously on most cell types and it is therefore difficult to assign distinct signalling functions to each receptor (Natoli *et al.*, 1998a; Orlinick and Chao, 1998). However experiments using *TNFα-/-* and *TNFR1-/-* knockout mice have similar phenotypes with markedly reduced sensitivity to LPS after D-galactosamine priming (Pfeffer *et al.*, 1993; Pasparakis *et al.*, 1996). Furthermore the *TNFα-/-* phenotype can be restored to that of wild type mice by the preparation of a transgenic mouse with human TNFα which can only bind TNFR1 (Pasparakis *et al.*, 1996). Such studies indicate a central role for TNFR1 in TNFα signaling. The role of TNFR2 appears to be more complex. Although there is evidence that it is capable of signalling independently of TNFR1, it is believed that its kinetics, having a rapid k_{on} and k_{off}, favour a ligand passing role increasing the local concentrations of TNFα for the TNFR1 (Tartaglia *et al.*, 1993; Papadakis and Targan, 2000; Orlinick and Chao, 1998).

In the absence of ligand, TNFR1 is kept in the inactive monomeric state by the 60kDa "silence of death domains" (SODD) (Jiang *et al.*, 1999). Binding of TNFα homotrimers causes dissociation of SODD and trimerisation of TNFR1, allowing the death domains of the cytoplasmic tails to aggregate. These death domains recruit effector molecules to begin the signalling cascade as TNFR1 has no intrinsic kinase activity. These effector molecules include:

- TRADD (TNFR associated death domain)
- FADD (Fas associated death domain)
- TRAF2 (TNFR associated factor 2)
- RIP (Receptor interacting protein)
    - and constitute the TNFR1 signalling complex (Figure 1.8).

The TNFR2 does not have a death domain but interacts directly with TRAF1 and TRAF2 to mediate its effects.

The multiplicity of signals which results from TNFα ligand binding and activation of its receptor can be split into two parts. The first is the initiation of apoptotic cell death via FADD/MORT1 recruitment and subsequent activation of caspase proteases. The second is the initiation of cell protection events to prevent the cell undergoing apoptosis and promoting cell survival. These are believed to centre around activation of NFκB via activation of NFκB inducing kinase (NIK) by the TNFR1 (or indeed TNFR2) complexes. There is conflicting evidence of the role of TRAF2 in the activation of NFκB. Dominant negative TRAF2 inhibits *in vitro* NFκB activation by TNFα (Jobin *et al.*, 1999; Natoli *et al.*, 1998a) whereas *TRAF2-/-* knockout mice have normal TNFα induced NFκB activation (Yeh *et al.*, 1997). There is likely to be tissue specificity to these mechanisms as is seen with the activation of NFκB in the intestinal epithelium and the fact that it is partially dependent on TRAF2 (Jobin and Sartor, 2000).

Furthermore, there are NFκB independent mechanisms of cytoprotection. This is demonstrated by the fact that selective molecular inhibition of TRAF2 causes more

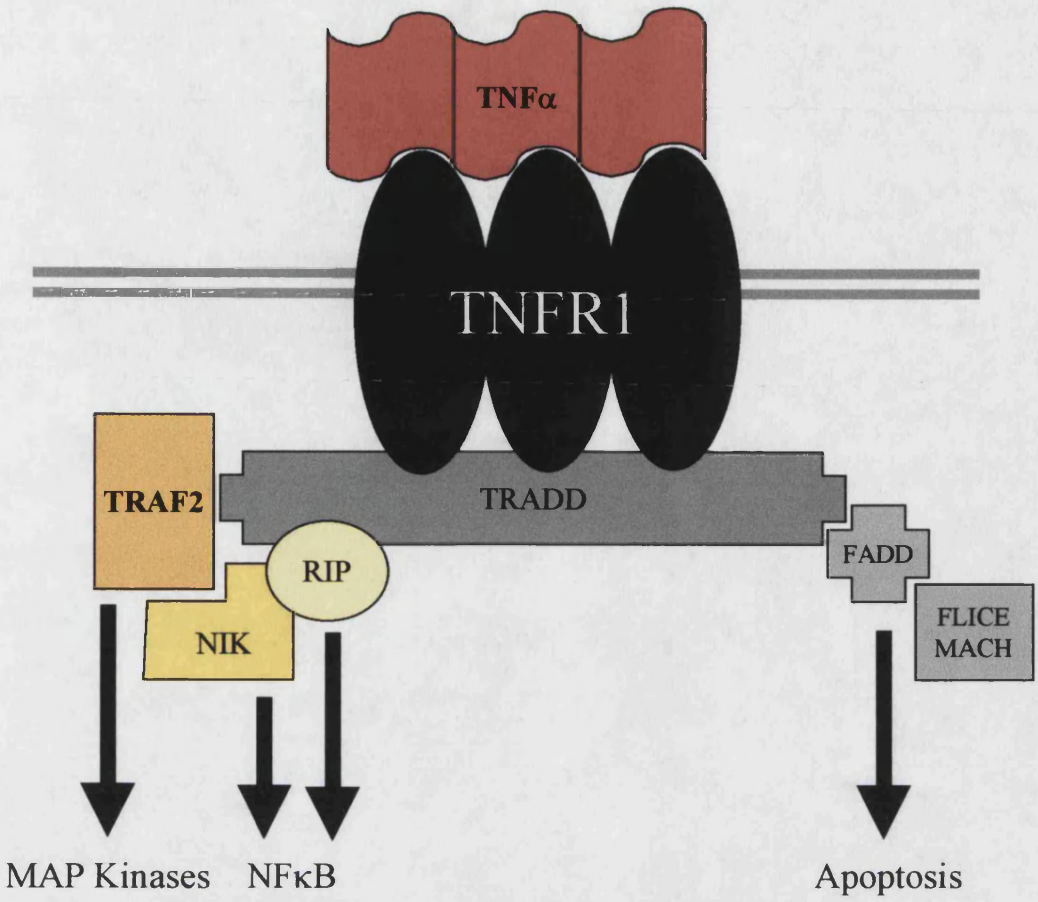


Figure 1.8: ***Signalling via the TNFα receptor***
Schematic representation showing the sequential, ligand induced, recruitment of different TNFR1 associated proteins and their subsequent downstream consequences

apoptosis than that caused by selective inhibition of NFκB (Natoli *et al.*, 1998b). However overexpression of TRAF2 is not sufficient to protect cells from apoptosis induced by TNFα and the protein synthesis inhibitor cycloheximide. Thus, there are TNFα induced cytoprotective mechanisms involved independent of both NFκB and TRAF2 and the complete picture remains to be fully elucidated (see Figure 1.9) (Natoli *et al.*, 1998a).

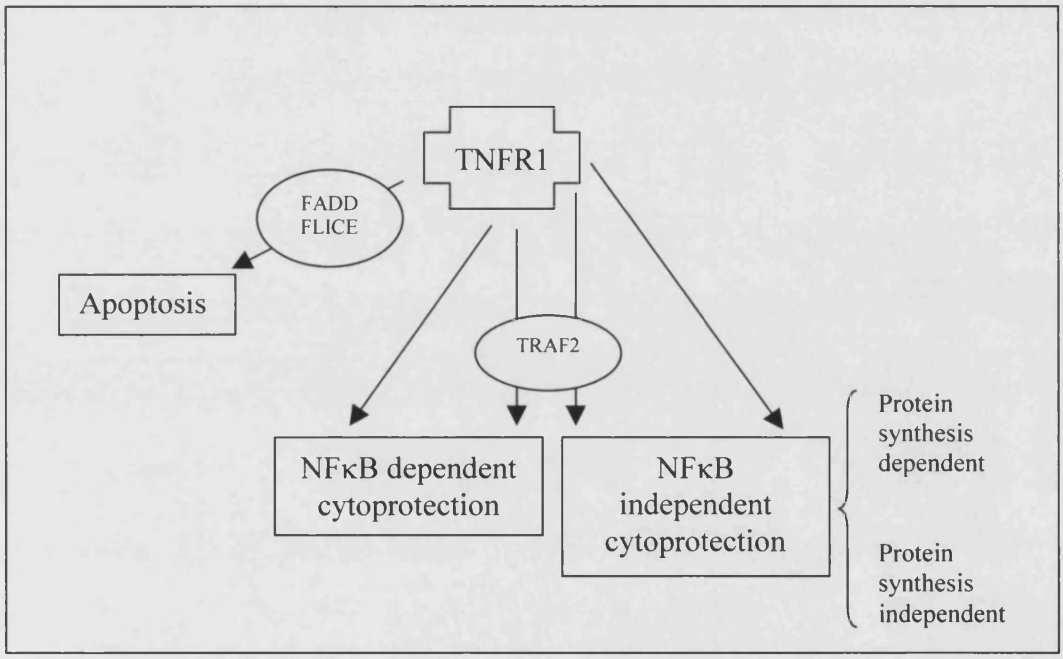


Figure 1.9: ***Consequences of TNFR1 activation***
A schematic representation of the TNFR1 dependent pathways promoting both apoptosis and cytoprotection

1.3.1.1 TNFα and Inflammatory Bowel Disease

There is strong evidence that TNFα plays a pivotal role in Crohn's disease, with its Th1 pattern of immune response. Inhibition of TNFα, with monoclonal antibodies targeted against it, gives an excellent therapeutic response in Crohn's disease which is not seen in ulcerative colitis (Targan *et al.*, 1997; Papadakis and Targan, 2000).

Furthermore, deletion of the 3′-UTR of the TNFα gene in mice resulting in an up-regulation of TNFα by stabilising its mRNA, results in an animal model showing enteritis with transmural inflammation and skip lesions very similar to Crohn's disease (Kontoyiannis *et al.*, 1999). This evidence of such a central role of TNFα in Crohn's disease is also supported by *in vitro* studies using IECs, macrophages and T cells which are all activated by TNFα as well as all being able to secrete TNFα. However, such *in vitro* work could not predict the great success of TNFα as a therapeutic target in Crohn's disease, compared to other possible targets such as IL-1.

1.3.1.2 TNFα and colorectal carcinoma

In contrast to the abundance of evidence of a role for TNFα in Crohn's disease there is little evidence or literature concerning TNFα and colorectal carcinogenesis and deductions have to be drawn from other tumour models. There is evidence in clinical trials that TNFα, when injected into and around tumours, can destroy neovasculature and thus slow growth (Eggermont *et al.*, 1996). In contrast to this, and perhaps more relevant, is evidence from an animal model of a different epithelial tumour. *TNFα-/-* knockout mice are resistant to the induction of skin tumours with DMBA (7,12-di-methylbenz[α]-anthracene), although there is no difference from the wild type in their subsequent malignant progression (Moore *et al.*, 1999). The malignant transformation occurring at sites chronic inflammation, seen in the skin and colon, may be due to ongoing prolonged exposure to TNFα.

1.3.2 Interleukin-1

Interleukin-1 (IL-1) is a highly inflammatory cytokine which can also upregulate host defences and act as an immunoadjuvant. It has two isoforms, IL-1α and IL-1β, which belong to a larger, evolutionarily conserved, family which includes fibroblast growth factor (FGF) and IL-18. Both forms of IL-1 are synthesised as 31kDa precursor forms which require processing by specific proteases to produce a mature 17kDa secreted cytokine. Although the biological activities of IL-1α and β are effectively indistinguishable, there are important differences between the two molecules.

ProIL-1α is secreted by cells when they die and is then cleaved by extracellular proteases (Kobayashi *et al.*, 1988). It has been demonstrated that IL-1α can be secreted in the absence of cell death *in vitro* (Watanabe and Kobayashi, 1994). However, the fact that IL-1α is not commonly seen in the circulation or in body fluids, except during severe disease, means that release during cell death is likely to be the common mode of production (Watanabe and Kobayashi, 1994).

In contrast IL-1β is released by living cells on stimulation and, like TNFα and COX-2 shares many features of an immediate early gene. The precursor form lacks biological activity and is converted to the mature form by the action of the cysteine protease (or caspase) IL-1β converting enzyme (ICE), although other inflammatory proteases may be able to catalyse the conversion in the absence of ICE (Fantuzzi *et al.*, 1997).

The IL-1 cytokines are tightly regulated in a unique fashion (Figure 1.10). The first mechanism involves the soluble IL-1 receptor antagonist (sIL1Ra) which is structurally related to IL-1α and β. This molecule is released by the cell upon stimulation and, like IL-1α and β, is able to bind to the IL-1R1. However this ligand binding does not result in signal transduction, even at concentrations 10^6 times greater than those needed for IL-1α or β. The exact mechanism for this lack of response is not certain although the IL-1R accessory protein (IL-1RAcP), which is necessary for signal transduction, does not associate to the IL-1R1 bound to IL-1Ra (Dinarello, 1997).

The second novel mechanism of regulation is the presence of a second IL-1R (IL-1RII) which acts as a decoy receptor or "IL-1 sink" in that it lacks the ability to transduce signals on ligand binding. This receptor, like IL-1R1, can also be released from the cell surface and bind to IL-1, again without producing a response. These sophisticated mechanisms at abrogating IL-1 responses, combined with the low numbers of IL-1R1 on primary as opposed to transformed cells, make the intense inflammatory response to IL-1 all the more surprising.

The IL-1R1 is the IL-1 receptor mediating functional responses and shares a 45% homology with the *Drosophila Toll* gene. When the membrane bound form binds to IL-1α or β it associates with the IL-1RAcP to form a signalling complex which lacks any intrinsic kinase activity. It does however have a binding site at amino acids 513-520 for IL-1R1 associated kinase (IRAK) which can phosphorylate a 100kDa substrate and is partly responsible for downstream activation of NFκB, MAP kinases, and activation of the AP1 transcription factor. However there is also

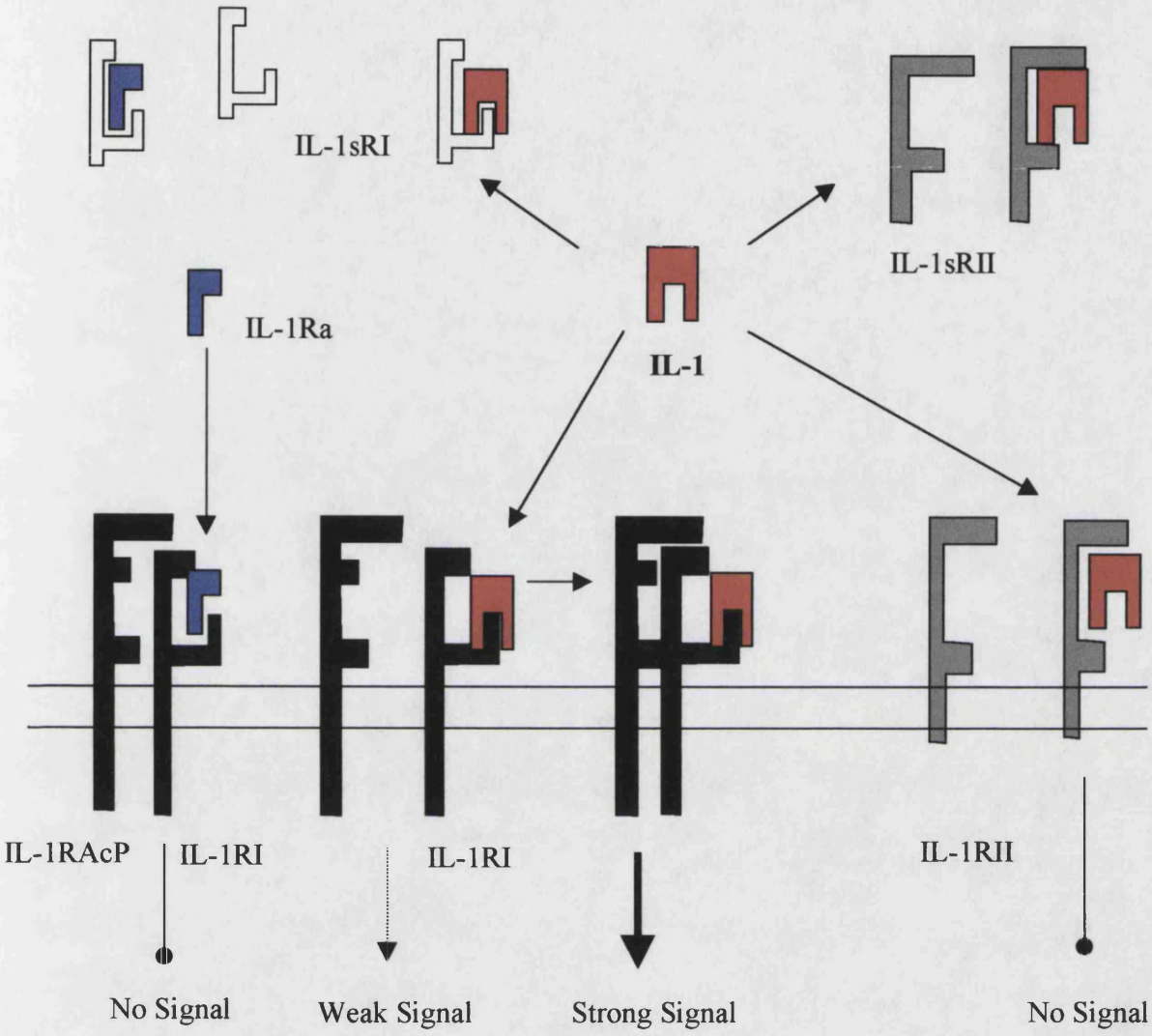


Figure 1.10: ***IL-1 Signalling***

Schematic representation showing the necessary interaction of IL-1R1 and the IL-RAcP to allow effective IL-1 signalling as a result of IL-1 binding. IL-1RII does not result in signal and neither does binding to either of the soluble forms of the two receptors. Finally IL-1 Receptor Antagonist (IL-1Ra) never results in signal transduction.

evidence that IL-1 can activate PI 3-Kinase and, as with TNFα, activation of NFκB may be PI 3-Kinase dependent (Reddy *et al.*, 1997).

1.3.2.1 IL-1 and Inflammatory Bowel Disease

IL-1 is raised in intestinal tissue of both Crohn's Disease and ulcerative colitis consistent with the chronic inflammatory process (Cominelli *et al.*, 1992; Fiocchi, 1998). Most interest has concentrated on the relative proportions of IL-1 to IL-1Ra with evidence that in those with IBD there is a bias favouring inflammation (increased IL-1:IL-1Ra ratio) (Cassini-Raggi *et al.*, 1995; Mahida *et al.*, 1996). However, despite the use of IL-1Ra in rheumatoid arthritis with clinical success (Jiang *et al.*, 2000), there has not been a concomitant breakthrough using such an agent in IBD (Cominelli *et al.*, 1990). There is also no clear information concerning the role of IL-1 in colorectal carcinoma.

1.3.3 Interferon γ

Interferon γ (IFNγ) is a Th1 immunomodulatory cytokine belonging to a family of interferons initially characterised by their antiviral actions. It is termed a type II interferon to distinguish it from the structurally distinct Type I interferons (IFNα and β). Human IFNγ is a non-covalent homodimer consisting of two 17kDa chains which can be variably glycosylated. It has a variety of anti-proliferative effects as well as upregulating MHC class I and inducing MHC class II as has been shown in IECs (Hoang *et al.*, 1992). IFNγ is induced by immune and inflammatory stimuli and is synthesised solely by T cells and natural killer cells.

There is only one known IFNγ receptor which is expressed on the majority of cell types and it is comprised of two subunits (Figure 1.11). The IFNGR1 (α chain or CD119w) is a 90kDa polypeptide with a Janus kinase (JAK)-1 binding site on its cytoplasmic tail, whereas the IFNGR2 (β chain or accessory factor-1) is smaller (62kDa) and has a JAK2 binding site. Both are required for signal transduction although IFNGR2 plays only a minor role in ligand binding. The receptor subunits do not associate in the absence of stimulation but do associate with JAK1 and JAK2. Ligand binding by IFNGR1 generates binding sites for IFNGR2 allowing association to form an active receptor complex. The inactive JAK1 and JAK2 molecules are brought into close association and activate each other by auto- and trans-phosphorylation (JAK2 → JAK1). Once activated, the receptor associated JAKs phosphorylate a functionally critical, tyrosine containing sequence ($_{440}$YDKPH$_{444}$) near the carboxy terminus of IFNGR1. This creates paired, ligand induced, docking sites for signal transducer and activator of transcription (STAT)-1. Two inactive STAT1 molecules bind via their SH2 motifs and are then phosphorylated by the receptor associated JAKs. Once phosphorylated, the STATs dissociate and form a homodimer which translocates to the nucleus and bind to gamma activated sequence (GAS) elements in IFNγ responsive genes stimulating transcription (see Figure 1.11).

Among the IFNγ primary response genes are members of the Interferon Regulatory Factor family of transcription factors (IRFs) (Mamane *et al.*, 1999). Members of this family, such as IRF1, can be induced by IFNγ and subsequently bind to IFN stimulated response elements (ISREs) in secondary response genes to mediate

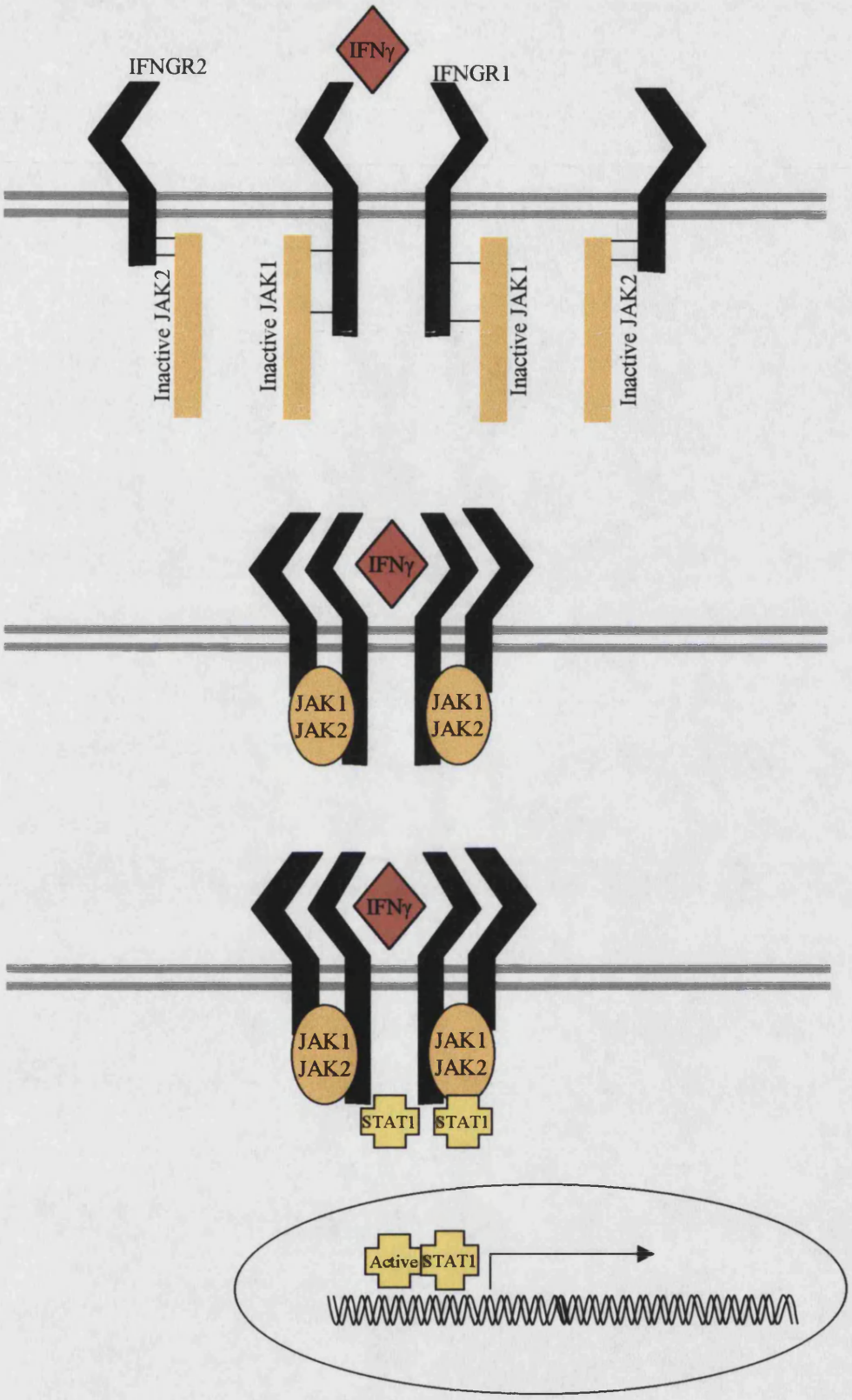


Figure 1.11: ***Signalling via the IFNγ receptor***
Schematic representation showing the sequential, ligand induced, activation of JAK2 and JAK1 resulting in activation and dimerisation of STAT1. This STAT1 homodimer translocates to the nucleus where it acts as a transcription factor. See text for further explanation and abbreviations.

further effects (Mamane *et al.*, 1999; Blanco *et al.*, 2000). Members of the IRF family can also be antagonistic or constitutively expressed and provide a complex level of regulation of IFNγ responses.

Work with chimeric receptors has demonstrated that the specificity of JAK1 and JAK2 to these events is due to their binding to the IFNGRs rather than substrate specificity (Kotenko *et al.*, 1996). There is evidence that this specific receptor association is crucial as cells lacking JAK1 or JAK2 are unable to activate STAT1 in response to IFNγ (Watling *et al.*, 1993; Muller *et al.*, 1993). STAT1 can be activated by other cytokines via other member of the JAK family. However, it does appear to be central to IFNγ responses with *STAT1-/-* knockout mice having a global deficit in IFNγ responses (Meraz *et al.*, 1996), although there is new evidence indicating that there may be some heterogeneity in different systems (Dupuis *et al.*, 2001).

1.3.3.1 IFNγ and Inflammatory Bowel Disease

IFNγ is spontaneously secreted by normal intraepithelial lymphocytes and lamina propria mononuclear cells and this is increased in IBD (Breese *et al.*, 1993; Carol *et al.*, 1998). Furthermore it has been shown to play an essential role in the $CD45RB^{hi}$ CD4+ T cell restored SCID mice model of IBD (Powrie *et al.*, 1994). These findings would fit with it driving a classical Th1 response, especially in Crohn's disease. However, a more complex role for IFNγ is hinted at by its accepted use as a treatment for chronic granulomatous disease, which shares features with Crohn's disease (The international chronic granulomatous disease cooperative study group., 1991). Also IFNγ has been used in a small trial for the treatment of Crohn's disease

(Debinski *et al.*, 1997). Despite this, it is generally regarded as playing a contributory role, particularly in Crohn's disease, although this has not been exploited as a therapeutic target.

Similarly to IL-1, there is little information concerning IFNγ and colorectal carcinogenesis.

1.3.4 Th2 anti-inflammatory cytokines

The T cell derived cytokines IL-4, IL-10 and IL-13 are classically regarded as anti-inflammatory cytokines which promote a Th2 immune response and are all believed to be involved in gastrointestinal tract physiology.

1.3.4.1 Interleukin-4 and Interleukin-13

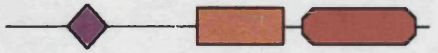
IL-4 and IL-13 share many similarities in functional response due to common receptor components and downstream signalling pathways. Indeed it has been proposed that IL-4 can mimic every IL-13 induced response (Callard *et al.*, 1996). The functional outcome of these cytokines is typified by the findings for IL-13 whose many anti-inflammatory actions include the down regulation of a variety of chemokines (IL-8, MIP-1α), pro-inflammatory cytokines (TNFα, IL-6, IFNγ), and other inflammatory mediators (nitric oxide) in systems such as LPS stimulated monocytes, intestinal epithelial cells, activated monocytes/macrophages or endothelial cells (Minty *et al.*, 1993; de Waal Malefyt *et al.*, 1993; Kolios *et al.*, 1996; Wright *et al.*, 1997). Furthermore, both cytokines have been shown to activate

PI 3-Kinase in macrophages (Montaner *et al.*, 1999). IL-13 has also been shown to activate PI 3-Kinase in intestinal epithelial cells and this mechanistic pathway appears to mediate the anti-inflammatory actions of this cytokine in this system (Wright *et al.*, 1997).

Both IL-4 and IL-13 are produced by T cells and monocytes/macrophages and are present in the normal colon (Carol *et al.*, 1998; Vainer *et al.*, 2000). Both have also been demonstrated as being decreased in IBD with IL-13 decreased in ulcerative colitis, especially when active (Vainer *et al.*, 2000), and IL-4 decreased in both ulcerative colitis and Crohn's disease (West *et al.*, 1996). These findings suggest an immunological deficit, congenital or acquired, which may predispose to IBD.

1.3.4.2 Interleukin-10

IL-10 is a pleiotropic cytokine which has attracted much interest in the context of IBD. The best evidence of its involvement comes from *IL-10-/-* mice which develop an enteritis with many features of IBD (Kuhn *et al.*, 1993). This is supported by *in vitro* studies showing that IL-10 is a potent suppressor of pro-inflammatory cytokine and chemokine generation by activated monocytes/macrophages (Moore *et al.*, 1993). It is therefore disappointing that targeting IL-10 in the treatment of IBD, as with IL-4 and IL-13, has not yielded more impressive clinical benefit (Van Deventer *et al.*, 1997).

Class	Structural Features of Catalytic Subunits	Subunits Catalytic	Subunits Adaptor	Structural Features of Adaptor Subunits	Regulation	Lipid Substrates
I A		p110α, β, γ	p85α p85β p55γ		Tyrosine Kinases and Ras	PtdIns PtdIns(4)P PtdIns(4,5)P_2
I B		p110γ	p101		Heterotrimeric G proteins and Ras	
II		PI3K-C2α, β, γ	?		Tyrosine Kinases? Heterotrimeric G proteins?	PtdIns PtdIns(4)P PtdIns(4,5)P_2
III		Vps34p Analogues	p150		Constitutive	PtdIns

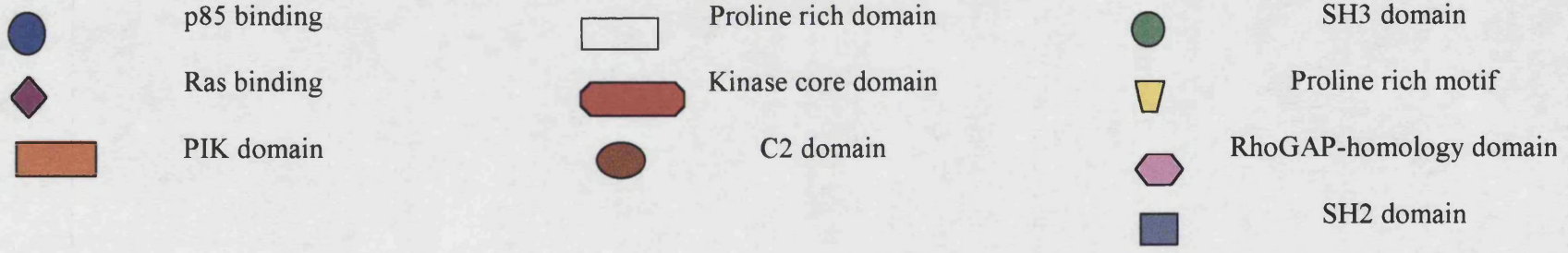


Figure 1.12: ***The PI 3-Kinase Family***
The different classes of mammalian PI 3-Kinases and schematic representations of their strucure

1.4 Signalling Pathways

1.4.1 Phosphatidylinositol 3-Kinases

1.4.1.1 PI 3-Kinases and their downstream effector targets

The term PI 3-Kinase is applied to a family of proteins that are able to phosphorylate the D-3 position of the inositol head groups of phosphoinositide lipids, namely phosphatidylinositol (PI), phosphatidylinositol(4)phosphate (PI(4)P) and phosphatidylinositol(4,5)bisphosphate ($PI(4,5)P_2$). This results in the formation of PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ respectively (Fruman *et al.*, 1998; Wymann and Pirola, 1998; Vanhaesebroeck and Waterfield, 1999;). PI(3)P is constitutively present in eukaryotic cells and its levels are largely unaltered upon cellular stimulation. In contrast, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are generally absent from resting cells, but their intracellular concentration rises markedly upon stimulation via a variety of receptors suggesting a second messenger function. PI 3-Kinases can be divided into three main classes on the basis of the *in vitro* lipid substrate specificity, structure and likely mode of regulation (Figure 1.12). Hence, the class I PI 3-Kinases phosphorylate PI, PI(4)P and $PI(4,5)P_2$, resulting in the formation of PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$. They also interact with Ras and form heterodimeric complexes with adaptor proteins that link them to different upstream signalling events. The prototypical class I_A PI 3-kinase is a heterodimer consisting of the 85kDa regulatory subunit (responsible for protein-protein interactions either via protein tyrosine phosphate-binding SH2 domains or SH3 domains and/or proline-rich regions) and a catalytic 110kDa subunit. The existence of multiple isoforms of

both components, which do not appear to preferentially associate, means that there is considerable scope for specific variation between tissues and with association to different receptor tyrosine kinases. The class I_B PI 3-Kinases are stimulated by G protein βγ subunits and do not interact with the SH2-containing adaptors that bind class I_A PI 3-Kinases. Instead, the first identified member of this family, p110γ, associates with a unique p101 adaptor molecule. The class II PI 3-Kinases (e.g. PI 3-Kinase-C2α) are characterised by the presence of a C-2 domain at the carboxyl terminus and utilise predominantly PI and PI(4)P as substrates, whereas the class III PI 3-Kinases utilise only PI as a substrate (e.g. mammalian PI 3-kinase and *Saccharomyces cerevisiae* Vps34p). The Mammalian homologue is likely to be the main source of PtdIns(3)P and has been shown to be expressed, along with class I_A PI 3-Kinases, in a colonic carcinoma derived intestinal epithelial cell line (Petiot *et al.*, 2000).

A number of proteins have been identified that directly bind $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ via PH domains including protein kinase B (PKB/Akt), $PI(3,4,5)P_3$-dependent protein kinase-1 (PDK-1), Bruton's tyrosine kinase, various PLC isoforms and exchange factors for the ADP-ribosylation factor family of GTP-binding proteins (Figure 1.13). Moreover, the D-3 phosphoinositide lipids have been linked to the triggering of a diverse array of cellular responses including cell survival, mitogenesis, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, superoxide production as well as actin polymerisation and chemotaxis.

Of these downstream effectors of PI 3-Kinases, PKB is of particular interest. It is a serine/threonine kinase that is itself activated by two distinct phosphorylation events,

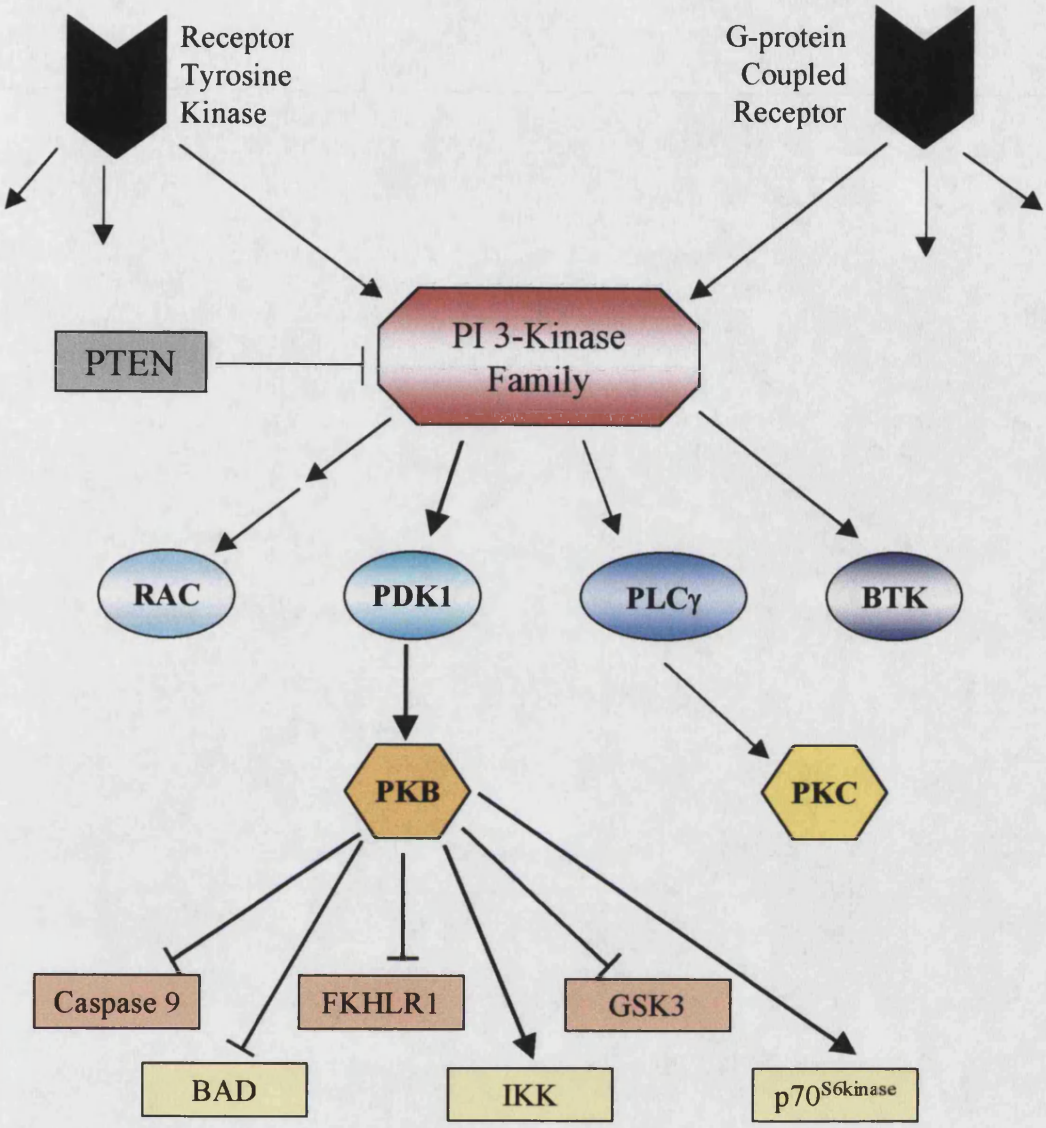


Figure 1.13: ***Downstream effectors of the PI 3-Kinase Family***
Schematic representation showing the activation of PI 3-Kinase by G protein coupled receptors and Tyrosine Kinase linked receptors. Binding to these receptors will also have other effects. Once activated PI 3-Kinase activates a number of downstream effectors which may in turn have a number of differing functional effects. PTEN Phosphatase and Tensin Homologue Deleted from Chromosome 10; PDK PI 3-Kinase dependent kinase; PLCγ phospholipase Cγ; BTK bruton's tyrosine kinase; PKB protein kinase B; PKC protein kinase C; FKHLR forkhead related ligand; IKK IκB kinase

and which probably both involve the upstream kinase PH domain-containing kinase PDK-1. PKB has now been demonstrated to phosphorylate a number of proteins, particularly those involved in regulating glucose metabolism and cell survival, reviewed in Datta *et al* (Datta *et al.*, 1999). In particular, PDK-1 and PKB as well as the PI 3-Kinase homologue, mammalian target of rapamycin (mTOR), contribute to the activation of $p70^{S6\ kinase}$, an enzyme thought to initiate protein synthesis.

1.4.1.2 PI 3-Kinases and Colorectal Carcinoma

There is now a growing body of evidence to support the notion that activation of PI 3-Kinases is associated with colorectal carcinoma and that this increased activity is playing an aetiological role. This evidence is considered below:

1. Development of colon cancers in the absence of 3'-phosphoinositide lipid phosphatases. One line of evidence comes from work on the tumour suppressor gene PTEN (phosphatase and tensin homologue deleted from chromosome 10). The PTEN gene product is a 3′ phosphatase which specifically degrades the major primary product of PI 3-Kinase activity, namely $PI(3,4,5)P_3$ (Maehama and Dixon, 1998; Cantley and Neel, 1999;). Germ-line mutations of PTEN occur in related, inherited, neoplastic disorders such as Cowden's syndrome, Lhermitte-Duclos disease and Bannayan-Zonana syndrome. These disorders share similar pathological traits including the formation of multiple benign tumours and an increased incidence of malignant cancers throughout the body, including the colon (Lynch *et al.*, 1997; Chi *et al.*, 1998). This is supported by work using *PTEN+/-* heterozygous mice which develop intestinal polyps which interestingly often cluster to a small part of the gastrointestinal tract (DiCristofano *et al.*, 1998; Podsypanina *et al.*, 1999).

2. Expression of PI 3-Kinases in colon cancers. The best characterised effector of the PI 3-Kinases and its 3′-phosphoinositide lipid products is PKB, which has a well-defined role in promoting cell survival and cumulative evidence indicates that the PI 3-Kinase/PKB pathway is oncogenic and involved in neoplastic transformation of mammalian cells. Thus, PKBβ is amplified and over-expressed in some ovarian cancers whilst PKBγ is overproduced in some breast cancers. However, to date there is no information as to whether PKB is up-regulated in colon cancers. Nevertheless, the suggestion of a causative role for PI 3-Kinases in colorectal cancer is supported by the fact that there is increased PI 3-Kinase activity in colorectal carcinoma specimens. Immunoprecipitation of whole colonic cancer tissue with specific antibodies to subunits of the p85/p110 heterodimer demonstrates significantly greater activity in *in vitro* lipid kinase assays compared to adjacent normal colonic mucosa (Phillips *et al.*, 1998; Benistant *et al.*, 2000). Similar increases in PI 3-Kinase expression and activity were detected in several adenocarcinoma cell lines compared to non-transformed fibroblasts (Benistant *et al.*, 2000). Moreover, micro-injection of neutralising antibodies to p110 catalytic isoforms into adenocarcinoma cell lines, revealed that both p110α and β play important roles in human colon cancer growth with a specific role for p110β in *de novo* DNA synthesis and an involvement of p110α in cell survival. It is of note that in this system p110γ inhibition had no effect on DNA synthesis or induction of apoptosis (Benistant *et al.*, 2000). However, the micro-injection studies are at odds with interesting data derived from knockout mice deficient in the class I_B PI 3-Kinase, PI 3-Kinaseγ. The *PI 3-Kinaseγ-/-* mice have been shown to spontaneously develop colorectal carcinomas (Sasaki *et al.*, 2000). This raises the possibility of conflicting roles of different

classes of PI 3-Kinases in the development of colorectal carcinoma, although it is worth noting that similar genetic constructs from other laboratories do not appear to demonstrate this phenotype (Hirsch *et al.*, 2000). However, the findings relating to development of carcinomas in *PI 3-Kinaseγ-/-* mice are also supported by work on human tumours and transformed cell lines. Accordingly, there is a decrease in p110γ expression in whole human tumour tissue compared to adjacent normal mucosa as assayed by western blotting. These workers also showed that transfection of three transformed adenocarcinoma cell lines with PI 3-Kinaseγ resulted in a decrease in their growth in both culture, and as tumours resulting from their subcutaneous injection into mice. However, confounding this was the finding that the same growth inhibition was seen with a kinase-dead mutant of PI 3-Kinaseγ. Hence, the inhibitory effects of PI 3-Kinaseγ on cancer growth may not be dependent on the catalytic activity of this isoform of PI 3-Kinase.

3. Effect of PI 3-Kinase inhibitors on tumour growth and intestinal epithelial cell apoptosis. Given the clear indication for a pivotal role of PI 3-Kinases in determining colorectal cancer, it begs the question as to what is the effect of PI 3-Kinase inhibitors on cancer cell growth? Indeed, pharmacological inhibition of PI 3-Kinases has also been demonstrated to inhibit the growth of several colon and ovarian carcinoma cells *in vitro* and also display anti-tumour activity in some human tumour xenograft models (Schultz *et al.*, 1995). Meanwhile, IL-13 was shown to abrogate cytokine-stimulated (e.g.TNFα/IL-1α/IFNγ) apoptosis of a colonic epithelial cell line via a PI 3-kinase dependent pathway (Wright *et al.*, 1999). Similarly, the Ras-induced down-regulation of the pro-apoptotic protein Bak has been shown to be sensitive to pharmacological inhibition of PI 3-Kinases (Rosen *et*

al., 1998). Together these data indicate that PI 3-Kinases are key signals responsible for the inhibition of apoptosis in intestinal epithelial cells (Wright *et al.*, 1999). However, these pharmacological experiments are limited by concerns about the specificity of available PI 3-Kinase inhibitors and the inability of such experiments to dissect the differing contributions of the classes of PI 3-Kinases to the biological read-outs employed.

4. PI 3-kinase and regulation of cell cycle progression. PI 3-Kinase activation is sufficient for cell cycle entry concomitant with cellular changes characteristic of oncogenic transformation (Klippel *et al.*, 1998). Indeed, upon activation of PI 3-Kinase, cells can enter S-phase as illustrated by activation of cyclin-dependent kinase (Cdk4) and Cdk2 and by the induction of DNA synthesis (Klippel *et al.*, 1998). Inhibition of PI 3-Kinase activity induces a senescence-like cell cycle arrest mediated by up-regulation of $p27^{Kip1}$ (Collado *et al.*, 2000), a cyclin-dependent kinase inhibitor, that is regulated by AFX, a member of the Forkhead family of transcription factors. These transcription factors, which also include FKHR and FKHRL, are directly phosphorylated by PKB, resulting in their export from the nucleus to the cytoplasm, where they form a complex with 14-3-3 proteins that effectively retains them in the cytoplasm away from their nuclear targets (Vanhaesebroeck, 2001). Hence, the induction of $p27^{Kip1}$ in response to inhibition of PI 3-Kinase activity appeared in conjunction with inactivation of PKB and consequent activation of the PKB substrate AFX (Collado *et al.*, 2000). Interestingly, an increase in PI 3-Kinase and PKB activity contributes to prostate cancer progression as demonstrated by an accelerated tumour growth and diminished AFX-forkhead-mediated transcription of $p27^{Kip1}$ (Graff *et al.*, 2000). It is tempting

to speculate therefore, that the observed increase in PI 3-Kinase activity observed in colorectal tumours (Phillips *et al.*, 1998; Benistant *et al.*, 2000) might also correlate with diminished $p27^{Kip1}$ transcription. To date however, there is no information available concerning the PI 3-Kinase-dependent regulation of $p27^{Kip1}$ in colonic carcinomas.

5. Transcriptional Regulation by PI 3-Kinases/PKB. So far, the role of PI 3-Kinases/PKB in colon cancer has been considered from the point of view of its well-defined role in promoting cell growth and survival. However, using fibroblasts and HEK293 cells, it is now emerging that PKB phosphorylates IKK-α, in response to TNF and PDGF. Although the precise mechanistic pathways mediating this effect remain contentious, this event in turn regulates IκB and ultimately leads to activation of the transcription factor NFκB which is involved in regulating transcription of a variety of genes important for a functional immune response (Ozes *et al.*, 1999; Romashkova and Makarov, 1999). This has been demonstrated to be relevant only in some cell types (Gustin *et al.*, 2001), but among the proteins whose expression is determined by NFκB is COX-2.

1.4.1.3 PI 3-Kinases and Inflammatory Bowel Disease

The role of PI 3-Kinases in IBD has received far less research interest than their role in tumourigenesis. Current theories are based on studies of non-gastrointestinal models of inflammation and *in vitro* models of intestinal epithelial inflammation and is summarised below:

Evidence for PI 3-Kinases Promoting Inflammation.

1. The f-Met-Leu-Phe (fMLP) induced oxidative burst in neutrophils is a PI 3-Kinase dependent effect. The response to this pro-inflammatory peptide is believed to be involved in the pathogenesis of IBD where neutrophils from patients with Crohn's Disease have significantly increased receptors for fMLP (Anton *et al.*, 1989). More recently, two studies using knockout mouse models showed that it is the class I_B PI 3-Kinaseγ which are involved in this response to fMLP (Li *et al.*, 2000; Hirsch *et al.*, 2000). Indeed, in a septic peritonitis model using *PI 3-Kinaseγ-/-* mice, there was severely defective macrophage accumulation indicating a crucial role for class I_B PI 3-Kinaseγ in chemotaxis and in successful inflammatory responses. Finally, it has recently been demonstrated that PI 3-Kinase products can bind the PX domain of phagocyte NADPH oxidase promoting reactive oxygen metabolite formation (Ellson *et al.*, 2001; Kanai *et al.*, 2001;).

2. The activation of PI 3-Kinases by the pro-inflammatory Th1 cytokine TNFα has been shown in a number of cell systems, including epithelial cells (Hanna *et al.*, 1999; Kim *et al.*, 1999; Pastorino *et al.*, 1999). However this has not been previously investigated in a gastrointestinal system.

3. It has been previously mentioned that the repertoire of responses of IECs includes chemokine secretion. Furthermore there is ample evidence that chemokines play a role in IBD and in trafficking and homing of intestinal lymphocytes. There is now a body of evidence derived from biochemical,

molecular and genetic studies that strongly supports the activation of PI 3-Kinases by chemokine receptors. PI 3-Kinaseγ is the primary lipid kinase activated by chemokines and is thought to be required for cell migration. However, there is substantial evidence for the activation of the p85/p110 heterodimer as well the class II PI 3-Kinase-C2α depending on the chemokine in question (Sotsios and Ward, 2000). There is thus, the potential that individual chemokine receptors can couple to one or more PI 3-Kinases and as such to different upstream activators and/or downstream effectors which one might expect to operate with different spatial and temporal characteristics.

Evidence for PI 3-Kinases Inhibiting Inflammation

1. In apparent conflict with the pro-inflammatory role of PI 3-Kinases in intestinal inflammation outlined previously, is the activation of PI 3-Kinases in intestinal epithelial cells by the Th2 cytokine IL-13 which is widely regarded as being anti-inflammatory (Wright *et al.*, 1997). This is supported by work in other systems demonstrating the activation of PI 3-Kinase by IL-13 and other Th2 cytokines including IL-4 and IL-10 (Mirmonsef *et al.*, 1999; Ceponis *et al.*, 2000). In the intestinal epithelium the activation of PI 3-Kinase by IL-13 was shown to be the mechanism mediating the inhibition of iNOS by this cytokine (Wright *et al.*, 1997). Although classically regarded as pro-inflammatory the functional role of iNOS in the context of IBD is contentious. This makes it difficult to comment on pro- or anti- inflammatory actions of PI 3-Kinase with respect to iNOS; suffice it say that it regulates an important enzyme known to be up-regulated in IBD.

2. A second likely anti-inflammatory action of PI 3-Kinase is its involvement in restitution – the migration of viable intestinal epithelial cells over an area of damage to restore epithelial continuity. This process is ongoing at times of damage such as in IBD. Using an *in vitro* wound model with intestinal epithelial cells, Insulin, Insulin-like growth factor-I (IGF-I) and a truncated analogue of IGF-I induced cell migration without altering proliferation. This process was inhibited by pharmacological inhibition of PI 3-Kinase, although there was no effect on restitution stimulated by phorbol 12-myristate 13-acetate (PMA) (André *et al.*, 1999). Human Intestinal Trefoil Factor (ITF) also promotes restitution and prevents the apoptosis that would occur with epithelial detachment in other circumstances. Elegant studies have dissected the molecular mechanisms involved in these processes (Kinoshita *et al.*, 2000; Taupin *et al.*, 2000). These showed that although, in this context, restitution is independent of PI 3-Kinases, the prevention of apoptosis occurring at this time is wholly dependent on activation of PI 3-Kinases and the downstream effector PKB or Akt. This in turn is dependent on the conformation and dimerisation of ITF. Despite their distinction between restitution *per se* and the prevention of apoptosis needed for restitution to occur, the pharmacological methods used do not shed any light on the classes or sub-classes of PI 3-Kinases involved.

1.4.2 Mitogen Activated Protein Kinases

The mitogen activated protein kinases (MAP kinases) are a major group of pathways which transduce extracellular signals to intracellular responses. The members of the MAP kinase pathways share core features concerning their consitutent kinases:

1. All MAP kinases are activated via phosphorylation of both Thr and Tyr in a Thr-Xxx-Tyr motif.
2. This activation is catalysed by a dual specificity serine-threonine MAP kinase Kinase (MAPKK) which, in turn, is phosphorylated and activated by an upstream MAP kinase kinase kinase (MAPKKK)
3. Once activated all MAP kinases can phosphorylate and activate other kinases or transcription factors, in the presence of ATP, to exert their biological effect

There are three main categories of MAP kinases – extracellular-regulated protein kinase (ERK or p42/44), p38 MAP kinase and c-Jun NH2-terminal kinase (JNK or p46/54) – and these shall be considered in turn below.

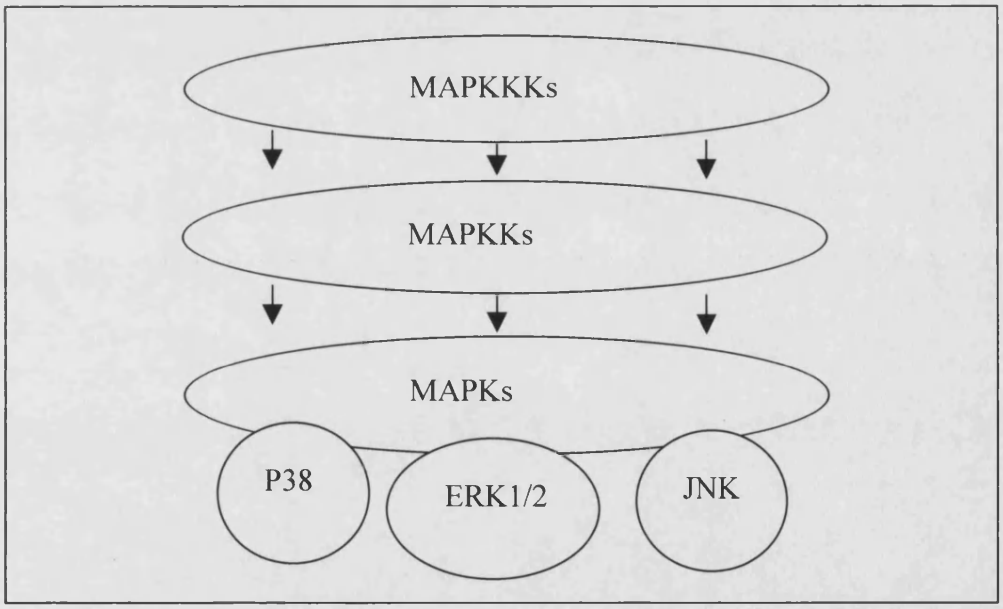


Figure 1.14: ***Schematic representation of the kinase cascades regulating MAP Kinase activation***

1.4.2.1 ERK

Extracellular-regulated protein kinase (ERK) was the initial classical MAP kinase and is involved in transducing signals from mitogens and growth factors involved in cellular proliferation and differentiation. Receptor ligand interaction promotes the recruitment of the small guanosine triphosphatase (GTPase) Ras, which then activates the serine-threonine kinase raf, which can activate members of the MAP kinase/ERK kinase (MEK) family. ERK1 (p44) and ERK2 (p42) are activated by MEK1 and MEK2 which are acting as MAPKKs. In this context raf is acting as a MAPKKK.

Downstream of ERK activation are its effectors, which include the transcription factors ATF2 and Elk, and the MAP kinase activated protein kinases (MAPKAPKs) 1 and 5. Study of the cellular effects of ERKs has been made possible by the use of small specific MEK1/2 inhibitors such as PD90859.

1.4.2.2 p38

The p38 MAP kinase is often grouped with c-Jun NH2-terminal kinase (JNK) as the stress activated protein kinases. They are both involved in regulating the cells responses to stress, inflammation and also apoptosis pathways. The p38 MAP kinase isoforms (α, β, β2, γ and δ) share a Thr-Gly-Tyr motif and are preferentially activated by pro-inflammatory cytokines (TNFα and IL-1), UV light, as well as heat and osmotic shock. There is some confusion over the precise details of the activating pathways for p38 MAP kinase which stems from the convergence of many upstream kinase dependent pathways (Herlaar and Brown, 1999; Ichijo, 1999;).

However p38 is activated by the MAPKKs MKK3 and MKK6 which act as points of convergence for MAPKKK pathways.

The effects of p38 activation are mediated by activation of transcription factors such as ATF2 and Elk1 as well as activating MAPKAPKs 2, 3 and 5. p38 activation plays a role in regulating development, inflammation and apoptosis, although this latter role is cell stimulus specific. The study of the functional consequences of its activation is assisted by the use of specific inhibitors of the pyridinyl imidazole family, such as SB203580 (Young *et al.*, 1997; Davies *et al.*, 2000).

1.4.2.3 JNK

There are many similarities between p38 MAP kinase and JNK. They are both activated by similar cellular stresses and have similar functional consequences. JNKs are phosphorylated on their common Thr-Pro-Tyr motif by MKK4 and MKK7 which, as with p38, integrate a wide variety of triggering stimuli (Davis, 1997).

Downstream of JNK activation and mediating its effects is activation of the transcription factors AP1, ATF2 and Elk1. The functional consequences of this are believed to be similar to p38 activation but research in this area is hindered by the lack of a specific inhibitor for JNK.

1.4.2.4 MAP kinases and the intestine

Despite the conceptual hypotheses that ERK may be involved in tumourigenesis, and that the stress activated protein kinases could play an important part in inflammation,

the data on clinical outcomes of MAP kinase activation has been confusing. ERK has been found to be raised (Licato and Brenner, 1998), unaltered (Miki *et al.*, 1999) and down-regulated in colorectal adenocarcinoma or adenomatous polyps (Attar *et al.*, 1996). p38 MAP kinase and, most consistently, JNK activity is seen to be increased in colorectal carcinoma (Licato and Brenner, 1998; Hardwick *et al.*, 2001;).

From the point of view of IBD there is no clear evidence of altered MAP kinase activation in either Crohn's disease or ulcerative colitis. However there is evidence, in *in vitro* models of intestinal disease, that MAP kinases do mediate pro-inflammatory mechanisms (Goke *et al.*, 1998).

1.5 Transcription Factors

1.5.1 Nuclear Factor κ B

Nuclear Factor κB (NFκB) is an inducible transcription factor first described as a B cell-specific factor that binds to a 10bp motif in the Ig κ light chain intronic enhancer (Sen and Baltimore, 1986a; Sen and Baltimore, 1986b). NFκB is a dimer of members of the Rel family of proteins with the prototype being a heterodimer composed of the RelA (p65) and NFκB1 (p50) subunits. This combination is the most potent gene activator among the NFκB family and is the major NFκB protein found in the nucleus of cytokine stimulated IECs (Jobin *et al.*, 1997).

1.5.1.1 Activation and regulation of NFκB

NFκB can be activated by a wide variety of stimuli which include:

- Cytokines and growth factors – such as TNFα, IL-1β and PDGF
- Bacteria and bacterial products – such as LPS, *H.pylori* and EPEC
- Mitogens – such as PHA and ConA
- Miscellaneous – such as oxidative stress and viruses including EBV and HBV

This activation is mediated by an elegant mechanism involving endogenous NFκB inhibitors belonging to the IκB family of proteins, which are characterised by an ankyrin repeat domain involved in protein/protein interaction. The prototypical member of this group is IκBα that binds to the p65 subunit of NFκB in the cytoplasm of unstimulated cells keeping it in an inactive state. On appropriate stimulation, there is activation of a complex of IκB kinases (IKK) which phosphorylate the Serine^{32} and Serine^{36} residues of IκBα. This step initiates the degradation, by ubiquitination, of IκBα which allows the release of free NFκB. This reveals its nuclear translocation signal resulting in transportation to the nucleus and NFκB dependent gene transcription. Among the many genes with a NFκB response element is the gene for IkBα which is subsequently resynthesised approximately 60-90 minutes after activation. This binds to the cytoplasmic NFκB resulting in its inhibition as shown in Figure 1.15.

However, all members of the IκB family do not follow this paradigm. IκBβ is not regulated by NFκB and does not have this intrinsic negative feedback resulting in

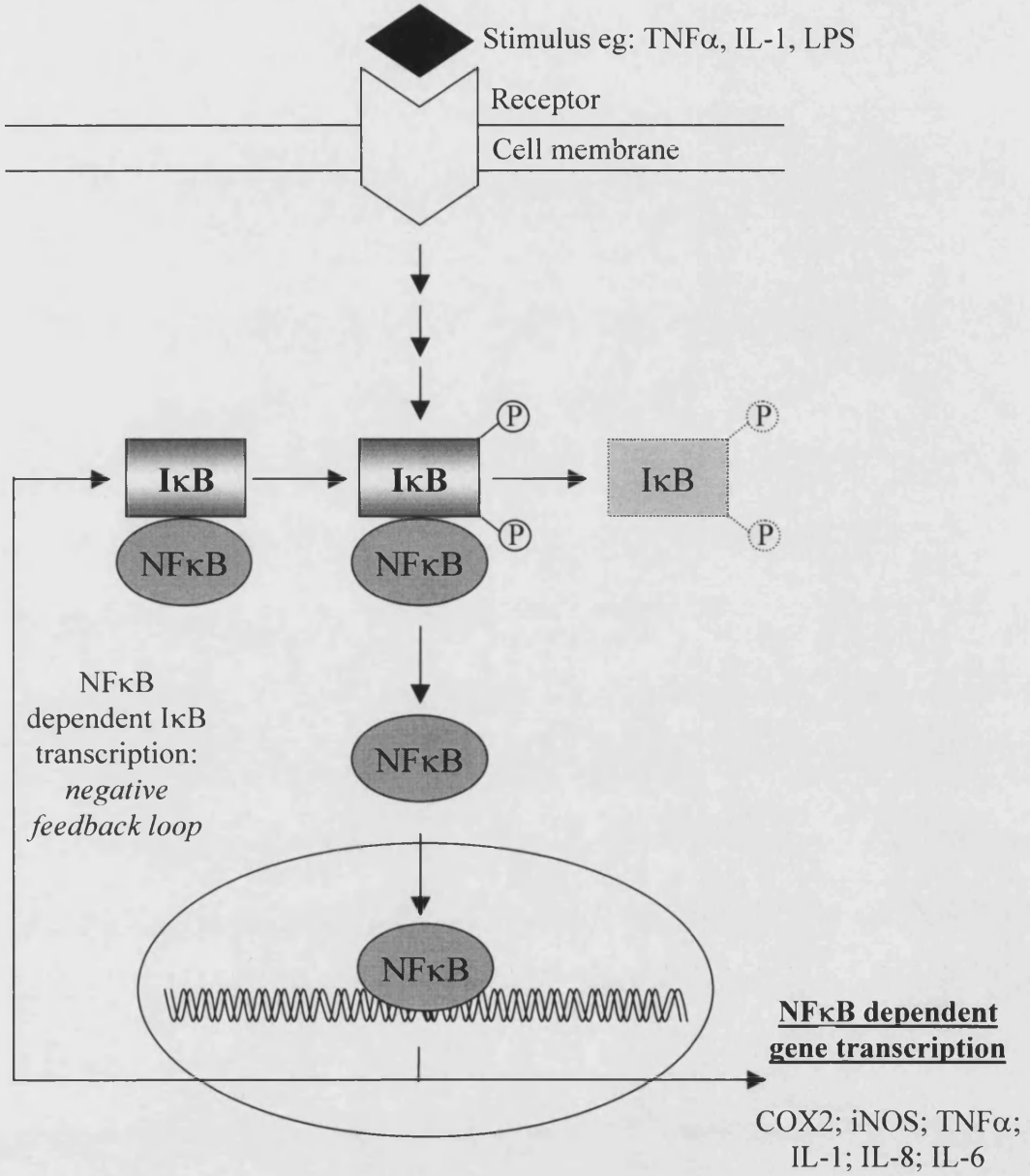


Figure 1.15: ***Activation of NFκB***
Schematic representation of the activation of transcription factor NFκB and its regulation, in a negative feedback loop, by IκB.

persisting activation. Furthermore there is evidence of post-translational modification of RelA phosphorylation state providing an alternative mechanism of regulating NFκB (Egan *et al.*, 1999).

Activated NFκB transcriptionally regulates a large number of genes including many immediate early (IE) genes. These include iNOS, IL-8, RANTES, TNFα, IL-1β, IL-6, ICAM-1 and COX-2. With regard to COX-2, its induction has been shown to be NFκB dependent in HT-29 intestinal epithelial cells using adenoviral transfection of a non-degradable form of IκBα to act as a specific NFκB inhibitor (Jobin *et al.*, 1998). Such NFκB inhibition had no effect on constitutive COX-1 levels. The genes which are transcriptionally activated by NFκB are generally pro-inflammatory and include cytokines that activate NFκB allowing potential amplification of the inflammatory response that needs to be tightly regulated.

1.5.1.2 NFκB and Inflammatory Bowel Disease

The general pro-inflammatory response to NFκB activation suggests an involvement in inflammatory diseases and also presents a potential target for anti-inflammatory therapies and this is particularly true for IBD. NFκB activation has been demonstrated in biopsy specimens, as well as lamina propria mononuclear cells (LPMCs) and primary IECs in both Crohn's disease and ulcerative colitis (Ardite *et al.*, 1998; Rogler *et al.*, 1998; Neurath *et al.*, 1996; Schreiber *et al.*, 1998).

It has also been demonstrated that many of the traditional treatments for IBD inhibit NFκB activation. In particular, the NFκB activation seen in biopsy samples from

people with IBD decreases with systemic steroid therapy (Ardite *et al.*, 1998). This does not rule out a bystander effect of NFκB activity decreasing with clinical improvement. Evidence against this is found in cases of Crohn's Disease of matched severity with those patients on steroids having decreased NFκB activation (Thiele *et al.*, 1999). There is also support from *in vitro* models where glucocorticoid treatment has been shown to decrease RelA binding activity, compared to those not receiving steroid, due to a physical interaction with the glucocorticoid receptor (McKay and Cidlowski, 1998; Adcock *et al.*, 1995). However this mechanism of glucocorticoid action is not universally seen with others reporting glucocorticoid induced impairment of IκBα degradation with no alteration of DNA binding (Hofmann *et al.*, 1998).

A similar picture is seen with the aminosalicylates. Sulphasalazine, a combination of sulphapyridine and 5-ASA, has been shown to be a specific inhibitor of NFκB activity by inhibiting phosphorylation of IκBα in colonic epithelial cells (Wahl *et al.*, 1998). It has also been shown to inhibit NFκB activation in T cells (Liptay *et al.*, 1999) and in both of these studies 5-ASA alone had no effect. In contrast, 5-ASA was seen to decrease NFκB mediated transcription in Caco-2 intestinal epithelial cells albeit at high concentrations (40mM) (Egan *et al.*, 1999) as well as inhibiting induced IκBα degradation in mouse IECs (Kaiser *et al.*, 1999). Although the general theme is of current therapeutic agents mediating their effects via NFκB, the multiple mechanisms of NFκB regulation, allied with the various differing assays to look at NFκB functional activity, mean that *in vitro* models may give conflicting results.

Attempts have been made to exploit NFκB as a therapeutic target for IBD. The best example of this was the use of a specific antisense oligonucleotide to RelA which, when locally administered to *IL-10-/-* knockout mice which spontaneously develop colitis, resulted in an abrogation of inflammation (Neurath *et al.*, 1996). An *in vitro* model of NFκB inhibition using a non-degradable IκBα has been delivered into IECs by adenovirus with marked inhibition of IL-8, iNOS and COX-2 (Jobin *et al.*, 1998). There has been much excitement about NFκB inhibition and this method of adenoviral delivery which unfortunately has yet to yield therapeutic benefits in humans.

1.5.1.3 NFκB and colorectal carcinoma

The role of NFκB in colorectal carcinogenesis has received far less interest than its more apparent inflammatory associations. Generally NFκB is regarded as having a protective action against apoptosis with *RelA-/-* knockout mice dying *in utero* of massive liver apoptosis (Beg *et al.*, 1995). This information has not been confirmed in IECs and, indeed there is conflicting evidence of a pro-apoptotic function (Giardina *et al.*, 1999). Finally there is evidence of NFκB having a negative influence on the cyclin dependent kinases thus inhibiting cell cycle progression (Hinz *et al.*, 1999). Thus it can be seen that NFκB may have a role in carcinogenesis but what that is in the colon is, as yet, far from clear.

1.5.2 Peroxisome Proliferator Activated Receptors (PPARs)

The first PPAR family member was discovered in 1990 and initially attracted only moderate interest because peroxisome proliferation, from which they get their name, is only of physiological importance in rodents and there were no identified human ligands (Issemann and Green, 1990). However, they are now recognised as important ligand dependent transcription factors, involved in differentiation, adipogenesis and the inflammatory response (Vamecq and Latruffe, 1999).

PPARs are members of the nuclear hormone receptor superfamily and have three currently identified isoforms – α, δ, and γ. PPARs are activated by a combination of ligand binding, interaction with the heat shock protein hsp72, and cellular signalling which alters the phosphorylation state of receptor. Once activated the receptor forms a heterodimer with a 9 *cis*-retinoic acid receptor, RXRα, and then can interact with the peroxisome proliferator response element (PPRE) in the target gene to promote transcriptional activation.

1.5.2.1 PPARα

PPARα is an important regulator of lipid metabolism although an important function in intestinal physiology is unrecognised. As with all PPARs a number of fatty acid derivatives bind and activate PPARα and these include the lipoxygenase products LTB_4 and 8-S-HETE (Yu *et al.*, 1995; Devchand *et al.*, 1996). The NSAIDs indomethacin, fenoprofen, ibuprofen and flufenamic acid also act as ligands as well as the fibrate class of hypolipidaemic drugs which exert their therapeutic effect via

this mechanism (Lehmann *et al.*, 1997). Once activated PPARα has been shown to inhibit COX-2 via a NFκB dependent pathway and has been hypothesised as having an anti-inflammatory action (Staels *et al.*, 1998). In contrast the potent PPARα ligand, WY-14,643, induced COX-2 protein in a breast epithelial cell line demonstrating the lack of concensus in this area (Meade *et al.*, 1999).

1.5.2.2 PPARδ

Little is known of the physiological role of PPARδ, also known as PPARβ, although it has been shown to act as a receptor for the COX product PGI_2 in a model of blastocyst implantation (Lim *et al.*, 1999). It is also emerging as having a potential role in colorectal carcinogenesis (DuBois, 2001) where its mRNA is seen to co-localise with that for COX-2 although the full implications of this finding are yet to become clear.

1.5.2.3 PPARγ

PPARγ has been found to be an important member of this family of receptors. It is an established regulator of adipocyte differentiation and has been targeted for novel diabetic therapies. It is known to bind to COX products PGA_2 and PGD_2 (Yu *et al.*, 1995), with the cyclopentenone derivatives of PGD_2 such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2) being potent activators (Forman *et al.*, 1995; Kliewer *et al.*, 1995). It also binds the NSAIDs indomethacin, fenoprofen, ibuprofen and flufenamic acid (Lehmann *et al.*, 1997) as well as the thiazolidinedione class of anti-diabetic drugs.

The details of this interaction with aspects of the COX pathway need further elucidation. PGJ_2 has been demonstrated to inhibit LPS induced COX-2 expression at the mRNA level in macrophage like cells (Inoue *et al.*, 2000) whereas PPARγ agonists as well as NSAIDs were shown to induce COX-2 mRNA and protein in epithelial carcinoma cell lines (Meade *et al.*, 1999). Whether this is due to differences in sole induction or regulating another agent is unknown and needs further investigation.

PPARγ and IBD: Soon after PPARγ ligands were identified, an anti-inflammatory response to their binding was identified. They were shown to suppress pro-inflammatory cytokine production by monocytes (Jiang *et al.*, 1998), as well as inhibiting macrophage activation, the latter being due to antagonising the activities of the transcription factors AP-1, STAT1 and NFκB (Ricote *et al.*, 1998). When this was pursued in a colonic model, PPARγ activation inhibited the production of MCP-1 and IL-8 in Caco-2 and HT-29 intestinal epithelial cells and the mechanism for this was prevention of IκBα degradation and hence inhibition of NFκB binding (Su *et al.*, 1999). Others have subsequently shown that PGJ_2 can inhibit NFκB in a PPARγ dependent and independent fashion (Straus *et al.*, 2000). In an animal model of colitis PPARγ agonists cause a marked abrogation of inflammation in mice receiving oral dextran sodium sulphate (DSS) (Su *et al.*, 1999). This approach is now being studied in patients with ulcerative colitis (Lewis *et al.*, 2000) and may be the mechanism for the known beneficial effects of fish oil in IBD.

PPARγ and colorectal carcinoma: As PPARγ is prominently expressed in the colonic epithelium, is regulated by eicosanoids and by exogenous dietary fatty acids,

and has an important role in cellular differentiation, the functions of PPARγ in colorectal carcinoma have been studied. PPARγ is expressed in the epithelial cells of human colorectal carcinomas although there is some debate about its role (DuBois *et al.*, 1998; DuBois, 2001). In the *Min* mouse model mimicking human FAP, oral thiazolidinedione drug caused an increase in the number of colonic polyps which developed (Lefebvre *et al.*, 1998; Saez *et al.*, 1998). However work in this model stands alone in suggesting a pro-neoplastic action of PPARγ activation. Work using human colonic carcinoma derived cell lines, both *in vitro* and injected into nude mice, show that PPARγ agonists decrease tumour growth and increase differentiation (Sarraf *et al.*, 1998). Furthermore, the same group in a different study identified a subset of human colorectal carcinomas that contain somatic mutations in PPARγ and proposed a causal role (Sarraf *et al.*, 1999). With the licensed clinical availability of pharmacological PPARγ agonists, the effect of PPARγ activation in human colorectal carcinomas should soon be clearer.

2 MATERIALS

Material	Source
β-Glycerophosphate	Sigma, Poole (UK)
[^{32}P]-Orthophosphate (8500-9120 Ci/mmol)	DuPont NEN (Stevenage, UK)
2,3-Diaminonaphthalene (DAN)	Lancaster Synthesis Ltd.
2-Mercaptoethanol	Sigma, Poole (UK)
3-[N-morpholino]-propane-sulfonic acid (MOPS)	Sigma, Poole (UK)
5-Aminosalicylic Acid (5-ASA)	Sigma Chemical, UK. It was diluted in 48mM Hydrochloric acid to give a concentration of 20mM and stored in aliquots at 4°C.
5'Digoxygenin labelled iNOS probe	R&D Systems (Abingdon,UK)
Absolute Ethanol	Hayman Ltd., Witham, UK
Acrylamide/bis acrylamide	Bio-Rad, UK
Adenosine triphosphate	Sigma, Poole (UK)
Agarose	Sigma, Poole (UK)
Ammonium persulphate	BDH, Poole (UK)
Blocking reagent (for Northern analysis)	Boehringer Mannheim (UK)
Bovine serum albumin (BSA)	Sigma, Poole (UK)
Bromophenol blue	BDH, Poole (UK)

cDNA for COX-1	Oxford Biomedical, USA
cDNA for COX-2	Oxford Biomedical, USA
Cell culture plastics	Nunc, UK
Chloroform	Fisons, Loughborough (UK)
Control Digoxigenin Labelled DNA	Roche, Lewes, UK
COX-1 antibodies	Santa Cruz, USA
COX-2 antibodies	Santa Cruz, USA
Diethyl pyrocarbonate (DEPC)	Sigma, Poole (UK)
DIG High Prime Digoxigenin Labelling kit	Roche, Lewes, UK
Digoxigenin chemiluminescent detection kit for Northern blotting	Boehringer Mannheim (UK)
Dimethyl sulphoxide (DMSO)	Sigma, Poole (UK)
Dithiothretol (DTT)	Sigma, Poole (UK)
Enhanced chemiluminescence detection kit for Western blotting (ECL)	Amersham International, UK
Ethidium bromide	Sigma, Poole (UK)
Ethylenediaminetetraacetic acid (EDTA)	Sigma, Poole (UK)
Flo-scint IV scintillation fluid	Canberra Packard (UK)
Foetal bovine serum (FBS)	Gibco BRL, Paisley, UK
Folch lipids	Sigma, Poole (UK)
Formaldehyde	BDH, Poole (UK)
Formamide	BDH, Poole (UK)
Glacial acetic acid	Fisons, Loughborough (UK)

Glycerol	Sigma, Poole (UK)
Glycine	Sigma, Poole (UK)
Goat anti-rabbit peroxidase conjugate	DAKO, Denmark
Hank's balanced salt solution	Gibco BRL, Paisley, UK
Hepes (1M liquid)	Gibco BRL, Paisley, UK
Hydrochloric acid	BDH, Poole (UK)
IFN-γ: human recombinant; specific activity > 2×10^7 U/mg	Boehringer Mannheim, UK; stored in aliquots at -70°C
IL-1α: human recombinant; specific activity 5×10^7 U/mg	Gift from Glaxo (Greenford, UK); diluted in sterile PBS + 0.25% (w/v) BSA and stored in aliquots at -70°C)
IL-13: purified from culture supernatants of stable transfected CHO cells (Minty *et al.*, 1993)	Gift from Dr. A. Minty (Sanofi Recherche, Labege, France); diluted in sterile PBS + 0.25% (w/v) BSA and stored in aliquots at -70°C
IL-4: human recombinant; specific activity > 1×10^7 U/mg	Genzyme and Pepratech, UK
Leupeptin	Sigma, Poole (UK)
Lithium chloride (LiCl)	Sigma, Poole (UK)
LPS	Sigma, Poole (UK) and was stored in aliquots at -20°C.
LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one)	Affiniti (Exeter, UK)

Maleic acid	Sigma, Poole (UK)
McCoy's 5A medium	Gibco BRL, Paisley, UK
Methanol	Fisons, Loughborough (UK)
Methylamine	Fisons, Loughborough (UK)
Molecular weight markers	Bio-Rad
N-(2-(Cyclohexyloxy)-4-nitrophenyl) methanesulfonamide (NS-398)	Research Biochemicals International (RBI), USA. It was diluted to 20mM in absolute ethanol and stored in aliquots at -20°C.
N-(3-(Aminomethyl)benzyl)acetamidine (1400W)	Alexis Corporation, UK. It was diluted to 20mM in MilliQ water and was stored in aliquots at -20°C
Neutral buffered formalin	Sigma, Poole (UK)
Nitrocellulose	Schleicher and Schuell
NP40	Sigma, Poole (UK)
Ovalbumin	Donated by Dr. M. Welham
Phosphate buffered saline	Gibco BRL, Paisley, UK
Phosphatidylinositol	Sigma, Poole (UK)
Phosphatidylserine	Sigma, Poole (UK)
PKB-α ^{473}Ser, polyclonal Ab	New England Biolabs, USA
PKB-α, polyclonal Ab	Santa Cruz, USA
PKB-α, polyclonal Ab	New England Biolabs, USA
PMSF	Sigma, Poole (UK)
Polaroid film (type 55)	Sigma, Poole (UK)

Ponceau S	Sigma, Poole (UK)
Propan-1-ol	Fisons, Loughborough (UK)
Propan-2-ol	Fisons, Loughborough (UK)
Propidium iodide	Sigma, Poole (UK)
Protein G beads	Sigma, Poole (UK)
RNase A	Sigma, Poole (UK)
RNAzol B	Tel Test, Texas, USA
Sarcosyl	BDH, Poole (UK)
Sodium azide	Sigma, Poole (UK)
Sodium chloride	Sigma, Poole (UK)
Sodium dodecyl sulfate (SDS)	Sigma, Poole (UK)
Sodium fluoride	Sigma, Poole (UK)
Sodium hydroxide	Sigma, Poole (UK)
Sodium molybdate	Sigma, Poole (UK)
Sodium nitrite	Sigma, Poole (UK)
Sodium orthovanadate	Sigma, Poole (UK)
TEMED	Sigma, Poole (UK)
Tetrabutylammoniumhydrogen sulphate	Fluka, Germany
Tissue culture reagents	Gibco BRL, Paisley, UK
TNF-α: human recombinant; specific activity 6×10^7 U/mg	Gift from Bayer (Slough, UK); diluted in sterile PBS + 0.1% (w/v) BSA and stored in aliquots at-70°C)
Trisodium citrate dihydrate	Sigma, Poole (UK)
Triton X-100	Sigma, Poole (UK)

Trizma base	Sigma, Poole (UK)
Tween-20	Sigma, Poole (UK)
Versene	Gibco BRL, Paisley, UK
Wortmannin	Sigma, Poole (UK)
X-OMAT film	Amersham International, UK

3 METHODS

3.1 Cell Culture Conditions

3.1.1.1 HT-29 cells

The human colonic epithelial cell line HT-29 was obtained from the European Collection of Animal Cell Cultures (ECACC). HT-29 cells are human colon adenocarcinoma grade II cells isolated from a primary tumour in a 44 year old Caucasian female (ECACC). They are a well characterised epithelial cell line and have characteristics of normal intestinal epithelium such as epithelial polarity, presence of the actin-biding protein villin and the occurrence of an enterocytic differentiation (Chantret *et al.*, 1988).

HT-29 colonic epithelial cells were routinely cultured in $80cm^2$ tissue culture flasks in McCoy's medium supplemented with penicillin (10u/ml), streptomycin (10μg/ml), fungizone (0.5μg/ml), and 10% (v/v) FBS (referred to as complete medium). Cultures were maintained at 37°C in an atmosphere of 5% CO_2. The medium was changed every 3 days. To subculture confluent monolayers, the medium was removed and the cells were washed 3 times with PBS (w/o Ca^{2+} and Mg^{2+}). Cells were then washed with a 3ml Trypsin-EDTA mixture of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA. The excess solution was removed and the cells were incubated for approximately 5mins at 37°C until the cells had detached from the flask. The action of trypsin/EDTA was inhibited by adding 10ml of complete

McCoy's medium and the cell suspension was centrifuged at 200g for 5min. The cell pellet was resuspended in complete medium and cell counting and viability were checked in a Neubauer haematocytometer after mixing with Trypan Blue (Sigma). Dead cells stained blue, due to the uptake of Trypan Blue. Cell viability was always greater than 95%. Cells were counted and then seeded at 2-3 x 10^4/ml of McCoy's complete medium, into 80cm^2 tissue culture flasks for further culture, into 6-well plates for northern experimental protocols, into 97mm petri dishes for western and ELISA experimental protocols and into 170cm^2 flasks for radioactive lipid labelling. Flasks and plates reached confluency after approximately 6 days. For storage, cells were resuspended at 4 x 10^6 cells/ml of freeze medium. The freeze medium contained 10% of dimethylsulphoxide (DMSO) (Sigma), 40% FBS, and 50% McCoy's medium. The cell suspension was transferred to cryotubes (Nunc) at 1 ml/tube, gradually cooled in vapour phase of liquid nitrogen overnight and then stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted at 37°C in a water bath, washed in McCoy's medium, resuspended in complete medium and cells from 1 cryotube were seeded into 80cm^2 tissue culture flasks in McCoy's medium, continuing as above.

3.1.1.2 Caco-2 cells

The human colonic epithelial cell line Caco-2 was obtained from the European Collection of Animal Cell Cultures (ECACC). It was established in 1974 from a colorectal adenocarcinoma in a 72 year old caucasian male (Rousset, 1986).

Caco-2 colonic epithelial cells were maintained in a similar fashion with a few distinctions. The medium used was Eagles Minimum Essential Medium (EMEM)

with Earles Balanced Salt Solution (EBSS). This was supplemented with 1% Non-Essential Amino Acids (NEAA) and 2mM Glutamine as well as penicillin (10u/ml), streptomycin (10μg/ml), fungizone (0.5μg/ml), and 10% (v/v) FBS. Cultured cells were maintained in similar conditions to HT-29 cells and were passaged in the same way. Caco-2 cells had a visibly different phenotype to HT-29 cells, were easier to detach with Trypsin-EDTA and grew slightly more slowly.

3.1.1.3 Primary Colonic Epithelial Cell Isolation

Colonic epithelial cells were isolated from biopsy specimens obtained at colonoscopy, and from colonic resection specimens, both obtained with ethical approval from the Royal United Hospital, Bath. The specimens came from patients with a normal colonoscopy, or from resection specimens for colorectal carcinoma when tissue away from the tumour was used. The specimens were collected in ice cold HBSS (pH 7.3) and transported to cell culture facilities. They were then washed three times for five minutes each in fresh HBSS to remove any visible debris. Once cleaned the mucosal layer was carefully dissected from the underlying submucosa and then cut into 2-3mm pieces. These mucosal pieces were then washed in Ca^{2+} / Mg^{2+} free HBSS three times for five minutes each. The cleaned mucosal pieces were then transferred to Ca^{2+} / Mg^{2+} free HBSS containing 1mM DTT and incubated for 15 minutes at room temperature with rocking. The mucosal pieces were then washed again in Ca^{2+} / Mg^{2+} free HBSS three times for five minutes each and then transferred to Ca^{2+} / Mg^{2+} free HBSS containing 1mM EDTA and incubated for 30 minutes at 37°C with rocking. The supernatant, containing intestinal epithelial cells, was collected and spun down at 200g for five minutes and the cell pellet resuspended in 3ml RPMI. These cells were then placed on a Percoll

centrifugation gradient (Percoll at 0%; 30%; 40%; 60%; and 100%) and spun at 250g for 30 minutes without a brake. The epithelial cells were removed from the 0%-30% interface and resuspended IEC culture medium. At this point they were counted and viability was assessed using trypan blue staining. They were then aliquoted into experimental points of 10^7 cells per point.

3.2 Experimental Protocol

HT-29 or Caco-2 colonic epithelial cells were cultured in the container appropriate to the planned experiment:

Assay	Culture Vessel	Cell Number HT-29	Cell Number Caco-2
Northern analysis	6 well plates	3.5 x10^6 cells	2 x10^6 cells
Western analysis	92mm petri dishes	10^7 cells	6 x10^6 cells
ELISAs	92mm petri dishes	10^7 cells	6 x10^6 cells
^{32}P Lipid labelling	170cm^2 flasks		

Twenty-four hours prior to the experiment the confluent monolayers were washed and cultured in FBS-free medium. Growth arrested cultures were then treated with fresh FBS-free medium and stimulated with the appropriate doses of either drugs, or cytokines, or vehicle controls for the times described in the results section. Supernatants were collected, centrifuged to remove cellular debris and stored at

-80°C until assayed. Total RNA and cellular proteins were extracted as described below. Cell counting and viability were routinely checked at the beginning and the end of the experiment, by phase microscopy and by trypan blue exclusion, using representative wells. Cell viability was always greater than 95%.

3.3 Northern Analysis

3.3.1 Overview

Northern analysis is a technique for directly assaying the total amount of a specific mRNA in a population of cells. Once cells have been incubated in the appropriate conditions, the cells are lysed and the RNA isolated, purified and quantified. This RNA is then separated according to its mass by migration, in an electric current, through an agarose gel. The RNA is then transferred from the gel to a nylon membrane which can be stored. At a later date the membrane can be incubated with a labelled piece of cDNA complementary to the RNA to be assayed. Once bound, the membrane can be washed and the amount of label (either Digoxigenin or [^{32}P]) can be assessed and is directly proportional to the total amount of RNA under investigation present. This method of assaying RNA levels was chosen as it represents a direct measure of RNA present in the cell.

The alternative method of assessing RNA in cells is based on the polymerase chain reaction and involves both the initial reverse transcription of RNA to cDNA followed by the amplification of the specific cDNA in question. These extra steps

make this a less direct assay of RNA based on certain assumptions and therefore northern analysis was chosen for this work.

3.3.2 Generation of Membranes

3.3.2.1 RNA isolation

RNA was isolated from colonic epithelial cells using RNAzol B (Biotecx). This is a refinement of previous methods and promotes the formation of complexes of RNA with guanidium and water molecules. It also abolishes the hydrophilic interactions of DNA and proteins allowing them to be efficiently removed from the aqueous phase. The RNA remains in the aqueous phase allowing its extraction. After the removal of cell cuture supernatant, 1ml of RNAzol B was added to the cells resulting in cell lysis. The sample was solubilised by passing the lysate through a pipette several times. The samples were then placed in a 1.5ml eppendorf and frozen for a minimum of 12 hours at –80°C. When thawed 100µl of chloroform was added to each eppendorf and the samples were shaken vigorously for 15 seconds. They were then kept at 4°C for 5 mins before being centrifuged at 12000g for 15mins at 4°C. When removed the homogenate had formed two phases – a lower blue phenol/chloroform organic phase and a colourless upper aqueous phase. The DNA and proteins were in the interphase and organic phase while the aqueous phase contained the RNA which was carefully pipetted off into a new eppendorf. 500ml of Isopropanol was then added to the aqueous phase and the samples stored at 4°C for 15mins. They were then centrifuged at 12000g for 15mins at 4°C and an RNA pellet was subsequently visible at

the bottom of the tube. The supernatant was removed and replaced with 1ml cold 75% ethanol and the samples were then stored at –80°C.

3.3.2.2 Sample preparation

Eppendorfs containing the RNA samples were defrosted and centrifuged at 12,000 g for 15 mins at 4°C. The supernatant was removed and again replaced with 1ml cold 75% ethanol and this wash step was repeated. The supernatant was removed and the pellets air dried in a fume cupboard for 1 hour. RNA pellets were resuspended in 50µl MilliQ water and kept on ice. RNA was quantified by measuring absorbance of 2µl RNA in 98µl MilliQ water at 260nm. The amount of RNA present in the samples (in µg) was calculated by:

A_{260} x dilution factor (50) x 40 x volume of remaining RNA solution in ml (0.048)

ODs were also read at 280nm and 230nm to assess the purity of RNA. A value of less than 2 for the OD_{260}:OD_{280} ratio indicated protein contamination. A low OD_{260}:OD_{230} ratio indicated guanidine contamination. 30µl of RNA sample buffer was added to 10µg RNA and the samples were vortex mixed and heated for 15-30 minutes at 80°C. The samples were cooled on ice and 2.5µl of bromophenol blue solution was added. Samples were mixed and briefly centrifuged (5secs) prior to loading on agarose gels.

3.3.2.3 Gel preparation and transblotting

1% agarose gel was prepared by dissolving 3.6g agarose (Roche) in 280ml MilliQ water and then heating in a microwave oven until the agarose dissolved. 18ml 20X

MOPS buffer and 65ml formaldehyde were added and the gel solution allowed to cool to ~ 60°C before pouring. The gel was set with three 15 lane combs using tanks purchased from Bio-Rad Scientific Instruments (Bio-Rad Laboratories, Hercules, CA, USA). After 40 mins, the gel was transferred to a submarine tank (Bio-Rad), which was surrounded with ice, and covered with cold 1X MOPS running buffer. 10μg RNA per lane was loaded and the gel run at constant current of 100mA, until the bromophenol blue band had migrated 5cm (~ 2.5 hours). The gel was placed under a UV light and the ethidium-bromide stained 18S and 28S ribosomal RNA bands observed to assess equal loading. The gel was photographed using a polaroid CU5 88-46 land camera (Genetic research instrumentation Ltd) and type 55 polaroid film (Sigma). The gel was cut at the bottom right hand corner to allow orientation, and then agitated gently in MilliQ water for 30-60 mins to remove formaldehyde, prior to transblotting. The blotting tank consisted of a glass plate suspended in a sandwich box, which was half filled with 20X SSC buffer. A wide strip of 3MM filter paper (Whatman, Maidstone, UK), placed over the glass plate and reaching down into the buffer solution at each end, acted as a wick. The gel was placed upside down on the filter paper and covered with a piece of positively charged nylon membrane (Roche), which had been briefly soaked in 20X SSC. All air bubbles were removed by rolling with a sterile glass pipette, before covering the membrane with three pieces of similar size 3MM filter paper and a stack of paper towels. A 500g weight was placed on top and left overnight to allow the RNA to transfer by capillary action. The RNA was fixed onto the nylon membrane by baking in a vacuum oven (Jouan) at 120°C for 20 mins. The membrane was sealed in a plastic bag and stored at room temperature prior to hybridisation.

3.3.3 Digoxigenin (DIG) labelled COX cDNA probes

3.3.3.1 Generation of Digoxigenin (DIG) labelled COX cDNA probes

The COX-1 and COX-2 cDNA (Oxford Biomedical, USA) were used as templates for the synthesis of smaller probes (c200bp) labelled with Digoxigenin using the DIG-High Prime method from Roche. This method was originally developed by Feinberg and Vogelstein (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984) and is based on the hybridisation of oligonucleotides of all possible sequences to the denatured cDNA to be labelled. The complementary DNA strand is synthesised by Klenow enzyme using the 3'-OH termini of the random oligonucleotides as primers. Modified deoxyribonucleoside-triphosphates labelled with Digoxigenin present in the reaction are incorporated into the newly synthesised complementary DNA strand. The DIG High Prime kit consists of a reaction mixture containing the Klenow enzyme and substrates such that DIG-11-dUTP is incorporated every 20^{th} to 25^{th} nucleotide in the newly synthesised DNA probes. To generate labelled probes either 10ng or 100ng of template cDNA for COX-1 or COX-2 was taken and added to MilliQ water to a final volume of 16µl. The cDNA was denatured in a boiling water bath for 10mins and then snap chilled at -80°C for 1 min. 4µl of the DIG High Prime reaction mixture was added, then mixed and centrifuged briefly before being incubated at 37°C for 20 hours. The reaction was then stopped by adding 2ml of 0.2M EDTA and by heating to 65°C for 10 min. By this method 10ng of template should yield 600ng of DIG labelled DNA probe and 100ng should yield 1500ng. Comparing the newly labelled probe to control DIG-labelled DNA using a dot blot method checked the efficiency of this labelling process. Serial

dilutions of the control DNA and the newly DIG-labelled probes were spotted onto a piece of nylon membrane used for northern blotting. This was then baked at 120°C for 20 mins and the membrane was developed using the hybridisation process described below.

3.3.3.2 Hybridisation with DIG-labelled cDNA COX probes

Hybridisation of membranes and detection of bound probes was performed essentially as described in the Digoxigenin (DIG) chemiluminescent detection kit for Northern blotting by Boehringer Mannheim. The DIG detection system is based on the labelling of nucleic acid probes with the steroid hapten, Digoxigenin. The DIG-labelled probes are then hybridised to membrane-bound RNA. Specific hybridisation is immuno-detected with an alkaline phosphatase conjugated anti-Digoxigenin antibody and visualised with the chemiluminescent substrate, CSPD, using X-ray film.

Sequential experiments were performed to optimise all the hybridisation conditions for each probe. These included performing "mock blots" on membranes without bound RNA to assess background levels of probe at differing concentrations and temperatures. After initial experiments COX-2 probes were hybridised at 42°C, while COX-1 probes were used at 50°C. Both probes were used at a final concentration of 25ng/ml.

The volumes specified are for a $100cm^2$ membrane and pre-hybridisation, hybridisation and wash steps were all performed at the appropriate hybridisation temperature. The membrane was prehybridized by incubating with 20ml high SDS

hybridisation solution for 1 hour in a sealed bag. The hybridisation solution was discarded and 2.5ml of probe, diluted to 25ng/ml in high SDS hybridisation solution, was added. The total cDNA probe needed (62.5ng/2.5ml) had already been boiled for 10 mins in 100µl of the high SDS hybridisation solution before being snap chilled for 1 min at -80°C and made up to the final concentration of 25ng/ml. This denatured the cDNA allowing it to bind to the target RNA on the membrane. All air bubbles were removed and the bag resealed and incubated overnight. The membrane was transferred to a small sandwich box and washed:

- twice for 15 min in 2X SSC / 0.1% SDS solution at the hybridisation temperature
- twice for 15 mins in 0.1X SSC / 0.1% SDS solution at the hybridisation temperature.

The probed membranes were then developed.

3.3.3.3 Hybridisation with DIG-labelled oligonucleotide probes

The methods for hybridisation using oligonucleotide probes were closely related to those for cDNA probes with the following alterations. Hybridisation temperature was optimised for β-actin probes and they were hybridised at 42°C at a final concentration of 10ng/ml. The standard hybridisation buffer used throughout did not need to have a high SDS content. Similarly, the oligonucleotide probes did not require boiling and then snap chilling prior to use. Otherwise the hybridisation steps were the same as those described above.

3.3.3.4 Visualisation of DIG-labelled probes

The following steps were then performed at room temperature on a shaking water bath. Membranes were washed for 10 mins in washing buffer, prior to blocking for 30 mins with 100ml buffer 2. Membranes were incubated for 30 min with 20ml alkaline phosphatase conjugated anti-DIG antibody diluted 1:10,000 in buffer 2. Membranes were then washed twice for 15 mins each in washing buffer and equilibrated for 5 mins in buffer 3. The membrane was drained and incubated for 5 mins between two plastic sheets with 1ml of lumigen PPD substrate diluted in 1:100 in buffer 3. The membrane was drained again, sealed in a plastic bag and incubated for 15 mins at 37°C in the dark. The membrane was then exposed to Kodak X-omat AR5 X-ray film (Sigma) for an appropriate time (1-4 hours) at room temperature.

After some time developing this technique, it eventually gave clear, interpretable results. However these subsequently became unpredictable despite repeated manipulation of experimental conditions, cDNA templates, solutions used and equipment and containers used for probing. This necessitated the development of an alternative method for specifically probing COX-2 cDNA.

3.3.4 [^{32}P]dCTP labelled COX-2 cDNA probes

3.3.4.1 Generation of [^{32}P]dCTP labelled COX-2 cDNA probes

This way of developing labelled COX-2 cDNA probes shares many similarities to the above described technique and involves the incorporation, using a random

priming method, of [^{32}P]dCTP into newly synthesised cDNA probes. After hybridisation these can be visualised directly using auto-radiography.

The same COX-2 cDNA was used as for the Digoxigenin method with 50ng being dissolved in 11µl MilliQ water in a screw-topped 1.5ml eppendorf. This was denatured by boiling for 10 mins and then spun down briefly. 4µl of High Prime enzyme (Roche) was added to this, followed by 5µl of [^{32}P]dCTP (Amersham, UK). The reaction mixture was briefly mixed, spun down and incubated at 37°C for 20 mins. Meanwhile an Elutip (Schleicher and Schuell, London, UK) was flushed very slowly with 2ml of High Salt solution followed by 5ml of Low Salt solution. After incubation, 1ml of Low Salt solution was added to the reaction mixture, aspirated and flushed and discarded slowly through Elutip. This process was then repeated to ensure all newly synthesised [^{32}P] dCTP labelled probe was bound to Elutip. Then, 1ml of High Salt solution was flushed through Elutip, eluting the labelled probe which was collected in the first 500µl passed. The second 500µl was appropriately discarded.

The efficiency of the labelling was assessed by counting the radioactivity emitted by the newly labelled probe. 1µl of the probe was placed in a scintillation vial and to this was added 5ml of scintillant (Flo-Scint IV, Packard, USA). This was then counted using a liquid scintillant counter (1209 Rackbeta, LKB Wallace, USA). The technique required $3x10^6$ counts per ml of hybridisation solution (ie: 21 $x10^6$ for a standard hybridisation tube with 7ml of hybridisation solution).

3.3.4.2 Hybridisation with [^{32}P]dCTP labelled cDNA COX-2 probes

Membranes for hybridisation were rinsed in 2X SSPE, then layered with Nylon sheets and placed in a hybridisation tube with a maximum of five membranes per tube. 7ml hybridisation solution was added to each tube and these were then pre-hybridised using a rotator in an oven at 63°C for 1-3 hours. Once the [^{32}P]dCTP labelled cDNA COX-2 probe was ready, an appropriate volume containing 21x10^6 counts was added to each tube. The tubes were then placed in the oven overnight for hybridisation. The next day the membranes were washed in their tubes:

- twice for 5 mins with 6X SSPE / 0.1% SDS at 37°C
- twice for 15 mins with 1X SSPE / 0.1% SDS at 37°C
- twice for 15 mins with 2X SSPE / 0.1% SDS at 63°C

The probed membranes were then visualised.

3.3.4.3 Visualisation of [^{32}P]dCTP labelled cDNA COX-2 probes

The probed membranes were removed from their hybridisation tubes and placed in plastic envelopes. All air bubbles were removed and the envelopes were then sealed. These envelopes were placed in clean phosphorimager cassettes which had been wiped by exposure to a light box for 10 minutes. Their co-ordinates within the cassette were noted and they were left for 3 days at room temperature. After this time the cassettes were opened in a dimmed room and placed in a phosphorimager plate reader (Phosphorimager SF, Molecular Dynamics, USA) which produced an electronic image of the membrane. This could be analysed using ImageQuant software (version 3), allowing measurement of band intensity, and the image could be converted to a .tif file for presentation. Alternatively the membranes could be

exposed to Kodak X-omat AR5 X-ray film (Sigma) for an appropriate time (3 days) at room temperature.

3.3.5 Stripping of Membranes

Membranes were stripped by washing twice in 0.1% SDS at 100°C while cooling to room temperature

3.4 Western Blot Analysis

3.4.1 Overview

Western analysis is a technique of assaying the relative amounts of specific proteins within cells. Once the cells have been incubated in the appropriate conditions the cells are lysed and the whole cell proteins are extracted and quantified. The whole cell extracts can then be run in a current through a polyacrylamide gel to separate the proteins according to their molecular weight. The proteins can then be transferred to a membrane which can be incubated with a primary antibody specific for the protein to be assessed. A second incubation with a labelled secondary antibody against the primary antibody is then carried out. The membrane is then washed and the relative amount of the labelled secondary antibody is then assessed, usually by using a horse radish peroxidase tag on the secondary antibody to catalyse a reaction which can then be visualised.

From the point of view of the primary antibodies that are used, they can be specific for certain conformations of the protein to be assayed. In particular they can be directed to a phosphorylated form of the protein where phosphorylation indicates a change in function such as activation. Such an antibody can then be used to assess relative changes in the amount of this phosphorylated form of the protein, and by inference is a surrogate marker of any associated change in function. To ensure that the total amount of the specific protein is unchanged the results from these phosphorylated antibodies are compared to results using an antibody against all forms of the specified protein, referred to as a pan antibody.

Finally, running whole cell extracts on a gel involves separating all the cell proteins and transferring them to a membrane. To allow only the protein of interest to be run on the gel, the whole cell extract can first be immunoprecipitated with an antibody to select only this protein. In this work, this was done for assaying protein kinase B (see 3.4.1.4)

3.4.1.1 Collecting samples

Attached monolayers of colonic epithelial cells were stimulated and incubated at 37°C as described. Stimulations were terminated at the appropriate times with aspiration of the supernatant, one gentle rinse with 5ml of ice cold PBS without Ca^{2+} or Mg^{2+}, and then the addition of 1ml ice cold lysis buffer. Cells were solubilised using a cell scraper and the resulting lysates were transferred to 1.5ml eppendorf tubes and incubated at 4°C for 15 mins. The samples were then centrifuged at

12000g for 5 mins at 4°C and the protein containing supernatant was transferred to a fresh eppendorf tube. Samples could be frozen at -80°C at this point.

3.4.1.2 Protein assay

Total protein per lysate was estimated using the Bio-Rad DC Protein Assay. This assay is based on the Bradford dye-binding procedure. Known concentrations of bovine serum albumin (BSA) diluted in lysis buffer were used as a standard curve. 5µl of sample or standard were placed in a 96 well plate with 25µl of working reagent A' (20µl reagent S into 1ml reagent A) and 200µl of reagent B. After 15min, the plate was read at 595nm on a Dynatech MR5000 platereader. The protein concentrations were calculated by linear regression from the standard curve and, if significantly variable, the lysate volumes were adjusted using the lysis buffer, thus ensuring equal concentrations of protein in each sample.

3.4.1.3 Whole cell extracts

An aliquot, usually 80µl, was transferred to a new eppendorf containing an appropriate volume (eg 20µl) of 5X SDS sample buffer. This was vortexed briefly, boiled for 5 mins and spun down before loading onto a gel. Samples could be frozen at -80°C at this point.

3.4.1.4 Immunoprecipitation for protein kinase B

For western analysis for protein kinase B (or Akt), immunoprecipitation was used rather than running whole cell lysates. 10µl of goat polyclonal Akt antibody (Santa

Cruz Biotechnology) was added to the remaining whole cell lysate, briefly vortexed and incubated for 1 hour at 4°C. 30µl of a 50% (v/v) suspension of protein G in PBS was then added and the mixture was incubated on a rotator for 2 hours at 4°C. At the end of this time the beads were captured by centrifugation at 12000g for 1 min at 4°C. The supernatant was aspirated and the pellet washed three times in lysis buffer with the immunocomplexes spun down between washes. After the last wash all the supernatant was aspirated and the pellet was resuspended in 24µl of 1X SDS sample buffer. This was vortexed briefly, boiled for 5 mins and spun down before loading onto a gel. Samples could be frozen at -80°C at this point.

3.4.1.5 Separation of cellular proteins by electrophoresis

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecule size. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (Laemmli, 1970). Proteins were separated by SDS-PAGE using the Bio-Rad Mini Protean II.

Minigels of the appropriate percentage were prepared as described in buffers and solutions section. The resolving gel was poured into the gel equipment and overlaid with MilliQ water. After the 20 mins for the resolving gel to set, the water was removed. The stacking gel was poured on the top of the running gel and set with a 10 or a 15 lane comb inserted. When the stacking gel was set the comb was removed, the wells were washed thoroughly with MilliQ water followed by running buffer. Then ~50µg of sample was loaded per lane (~10-20µl) alongside a molecular

weight marker. Gels were run at room temperature at 80V, until the bromophenol blue tracking dye entered the running gel. The voltage was then increased to 150 volts. Gels were run until the bromophenol blue band had reached the bottom of the resolving gel. Gels were then placed into transfer buffer.

3.4.1.6 Semi-dry transfer of proteins to nitrocellulose

The graphite electrodes of the semi-dry transfer apparatus (Pharmacia-Biotech Multiphor II) were dampened with semi-dry transfer buffer. This was followed by placing a sandwich of four pieces of 3MM Whatman paper cut to the size of the gel, one piece of nitrocellulose membrane, the gel and another four pieces of 3MM Whatman paper, all soaked in transfer buffer. Each layer was rolled gently to expel air bubbles. The transfer was run for one hour at 0.8mA/cm^2 of membrane (~40mA per gel). The membrane was then stained with Ponceau S to check for transfer and equal loading of the samples and also to confirm the location of the molecular weight markers. The stain was removed by washing the membrane in distilled water for 2 mins, followed by a 10 mins wash in Tris buffered saline (TBS).

3.4.1.7 Immunoblotting of nitrocellulose bound protein

Non-specific binding sites on the nitrocellulose were blocked by overnight incubation of the membrane with the appropriate blocking buffer at room temperature on a rocking platform. The nitrocellulose membrane was washed for 10 mins in TBS and then incubated with the primary antibody, made up in a 1:5 dilution of fresh blocking buffer in TBS, for 2 hours – overnight. The membrane was then washed on a rocking platform at room temperature:

- one 10 min wash in TBS
- three times for 10 mins in TBSN
- one 10 mins wash in TBS

The membrane was then incubated for 1 hour with the appropriate secondary antibody diluted in TBSN (0.1µg/ml usually equivalent to 1:10000). After this incubation washings were repeated as previously before a final wash for 10 mins in TBSN. 10mls of Enhanced Chemiluminescent Lumigen (ECL) reagent was added for 1 min and the membrane was then exposed to X-ray film for 0.5 – 30 mins. The film was developed using an RGII Fuji X-ray film developer.

3.4.1.8 Membrane stripping

Where appropriate, blots were stripped of bound antibody and reprobed with a different primary antibody. After the ECL procedure described above, the membrane was washed once in TBS for 10 mins and then placed in 50ml of stripping buffer in a sealed sandwich box and incubated at 55°C for one hour. After extensive washing in at least three changes of TBSN followed by a TBS wash, the membrane would be reblocked in the appropriate blocking buffer before the addition of the new primary antibody.

3.5 p38 MAP kinase assay

3.5.1 Overview

This assay to measure p38 MAP kinase activity uses a kit from Cell Signaling Technology. A monoclonal phospho$^{Thr180/Tyr182}$-specific antibody to p38 MAP kinase is used to selectively immunoprecipitate active p38 MAP kinase from cell lysates. The resulting immunoprecipitate is then incubated with ATF-2 fusion protein in the presence of ATP and kinase buffer, which allows immunoprecipitated active p38 MAP kinase to phosphorylate ATF-2. Phosphorylation of ATF-2 at Thr 71 is measured by western blotting using a phospho^{Thr71}-specific antibody and acts as a marker of p38 MAP kinase activity.

3.5.1.1 Collecting Samples

Attached monolayers of HT-29 cells were stimulated and incubated at 37°C as described. Stimulations were terminated at the appropriate times with aspiration of the supernatant, one gentle rinse with 5ml of ice cold PBS without Ca^{2+} or Mg^{2+}, and then the addition of 0.5ml 1X ice cold cell lysis buffer plus 1mM PMSF. The dish was then incubated on ice for 5 mins before cells were scraped off, transferred to microcentrifuge tubes and centrifuged at 4°C for 10 mins at 12000g. The protein containing supernatant was transferred to a fresh eppendorf tube. Samples could be frozen at -80°C at this point.

3.5.1.2 Immunoprecipitation using immobilised phospho$^{180/182}$ p38 antibodies

To 200µl of cell lyate was added 20µl of resuspended immobilised phospho$^{Thr180/Tyr182}$-specific p38 MAP kinase monoclonal antibody. This was incubated in a rotator at 4°C overnight. The next day the mixture was centrifuged at 12000g for 1 min at 4°C. The pellet was washed twice with 500µl of 1X cell lysis buffer and kept on ice. This was followed by two washes with 1X kinase buffer also keeping the pellets on ice.

3.5.1.3 Kinase assay

The washed pellet was suspended in 50µl of 1X kinase buffer supplemented with 200mM ATP and 2µg ATF-2 fusion protein and incubated for 30 mins at 37°C. The reaction was terminated with the addition of 12.5µl of 5X SDS sample buffer. The sample was boiled for 5 mins, vortexed briefly and then centrifuged at 12000g for 2 mins.

3.5.1.4 Western analysis

The sample was loaded onto a 10% SDS-PAGE gel and run as described above. The resulting gel was routinely transblotted and the subsequent membrane blocked overnight before incubation with the phospho^{Thr71}-specific antibody, all as described above.

3.6 Accumulation of D-3 Phosphoinositide lipids in intact cells

3.6.1 Overview

In order to assay PI 3-Kinase activity the accumulation of its D-3 phosphoinositide lipid products was assayed. Cells are initially washed with phosphate free media and then incubated with a radiolabelled phosphate donor. Aliquots of cells are then stimulated as appropriate and subsequently the lipids are extracted. This lipid fraction is then deacylated to render the glycerophosphorylinositol derivatives water soluble, and run on an HPLC column to separate the lipids and quantify them by their relative radioactive emissions.

3.6.1.1 Sample preparation

HT-29 cells were dissociated from the 170cm^2 flask using 4ml of Trypsin-EDTA mixture (0.05% (w/v) Trypsin and 0.02% (w/v) EDTA). They were then washed three times in phosphate-free Dulbecco's Modified Eagles Medium (DMEM) with 10 mins incubations at 37°C before each spin at 400g for 5 mins. The cells were subsequently resuspended in 10ml phosphate-free DMEM containing 10% dialysed foetal bovine serum and 20mM HEPES at 10^7 cells/ml and incubated at 37°C for 4 hours with 1mCi of [^{32}P]-orthophosporic acid (NEN).

After incubation, the cells were washed three times in phosphate-free DMEM and resuspended in McCoy's 5A (with 20mM HEPES, but without FBS) at 10^7 [^{32}P]-labelled cells per 120µl. Each aliquot of 120µl was placed in a 1.5ml screw topped

1.5ml eppendorf tube and equilibrated at 37°C for 10 mins. Each point was stimulated with 12µl of agonist or vehicle for the appropriate times and the reaction quenched by the addition of 700µl of ice cold chloroform/methanol/water (32.6%/65.3%/2.1%). The samples were then immediately placed on ice. 200µl of chloroform containing 10µg of Folch lipids as a carrier protein were then added along with 200µl of 2.4M HCl / 5mM tetrabutylammoniumhydrogen sulphate (TBAS). The extraction mixtures were vortexed and centrifuged at 3000g for 5 mins. The lower phase was removed and added to 400µl of 1M HCl / 5mM EDTA. The mixtures were vortexed again and centrifuged at 3000g for 5 mins. The lower phase was removed to screw topped 1.5ml eppendorfs with pierced lids and dried *in vacuo* using a Savant SpeediVac. When dried down, 1ml of 25% (v/v) methylamine/methanol/N-butanol (4/4/1) was added to the residue and, after vortexing, the samples were incubated in a 53°C water bath for 40 mins. This deacylation procedure renders the glycerophosphorylinositol derivatives of PI (3) *P* (GroPI (3) *P*), PI (3,4) P_2 (GroPI (3,4) P_2), PI (3,4,5) P_3 (GroPI (3,4,5) P_3) water soluble. The samples were then cooled for 1 min on ice and then dried *in vacuo* as before. Finally 500µl of water and 600µl of N-butanol/40-60% petroleum ether/ethyl formate (20/4/1) were added. The samples were vortexed and centrifuged at 750g for 30 secs. The upper phase was removed and discarded and the lower phase was dried *in vacuo*. The samples were then stored at -20°C until analysis.

3.6.1.2 HPLC analysis of samples

Anion exchange high performance liquid chromatography (HPLC) was used to analyse the lipid content of the samples using a water and phosphate buffer gradient

(Stephens *et al.*, 1989). The samples were resuspended in water and injected onto a Partisphere SAX column. The eluant was detected using a Canberra Packard A-500 Flo-One on line *beta* radiodetector where it was mixed in a ratio of 1:3 with Flo-Scint IV scintillation cocktail, according to the manufacturer's specifications. The results were analysed on a Flo-one data program. The retention times were compared to standards of [^{3}H] PI (4) *P* and of [^{3}H] PI (4,5) P_2. The identity of the various peaks obtained using this separation technique has been defined previously (Ward, 2000). The PI (3) *P* elution time was compared to that determined by Dr S.G. Ward by immunoprecipitating PI 3-Kinase using an anti-p85 antibody and performing an *in vitro* lipid kinase assay. The PI (3) *P* was isolated by thin layer chromatography (TLC) and then extracted from the TLC plate and its elution time on the HPLC monitored (personal communication). The elution times of the other 3-phosphorylated lipids were compared to those quoted in the literature (Stephens *et al.*, 1989; Ward, 2000). The levels of PI were used as an internal standard to confirm that each sample contained a similar amount of radioactivity, as the PI pool should not vary upon agonist stimulation.

3.7 Prostaglandin E_2 ELISA

3.7.1.1 Overview

This assay to measure PGE_2 concentration uses a kit from R&D Systems (Abingdon, UK). It is based on the competitive binding technique in which PGE_2 present in the sample competes with a fixed amount of alkaline phosphatase-labelled PGE_2 for sites on a mouse monoclonal antibody. During the incubation, the mouse

monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. The intensity of the colour generated is inversely proportional to the concentration of PGE_2 in the sample (Figure 2.1).

3.7.1.2 Assay procedure

Known concentrations of a PGE_2 standard were diluted in cell culture medium and used for a standard curve (5000pg/ml to 39pg/ml) which gave a quoted assay sensitivity as less than 36.2pg/ml. 100µl of standard or cell culture supernatant sample were placed in microplates coated with goat anti-mouse antibody. To each was added 50µl of PGE_2 conjugate (conjugated to alkaline phosphatase) and 50µl of PGE_2 antibody solution (mouse monoclonal). The plates were covered and incubated on a shaking platform for two hours at room temperature. After this incubation the wells were aspirated and washed with 200µl wash buffer using a multi-channel pipette. This was repeated to give a total of three washes and after the last wash the microplates were inverted and blotted against clean paper towelling. 200µl of pNPP substrate was then added to all wells and incubated at room temperature without agitation. The optical density at 405nm was determined immediately using a Dynatech MR5000 plate reader. The PGE_2 concentrations were calculated by comparison to the sigmoid standard curve using BioLinx software.

3.8 Prostaglandin D_2 ELISA

3.8.1.1 Overview

This assay to measure PGD_2 concentration uses a kit from Cayman Chemicals (Ann Arbor, MI, USA). It begins by converting the relatively unstable PGD_2 to stable PGD_2-methoxime (PGD_2-MOX) by methoximation (Figure 2.2). It is then based on the competitive binding technique in which the PGD_2-MOX now present in the sample competes with a fixed amount of acetylcholinesterase-labelled PGD2-MOX for sites on a specific rabbit antibody (Figure 2.1). During the incubation, the rabbit anti PGD_2-MOX antibody becomes bound to the mouse anti-rabbit monoclonal antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution (Ellman's reagent) is added to the wells to determine the bound enzyme activity. The intensity of the colour generated is inversely proportional to the concentration of PGD_2 in the sample.

3.8.1.2 Assay procedure

Firstly the methyl oximating reagent was prepared by diluting supplied methoxylamine HCl in 10ml of a 10% (v/v) solution of ethanol in water. To this was added the supplied sodium acetate to give the final reagent. The PGD_2 standard was then diluted in 900µl of MilliQ water and a 1:1 solution was prepared with the methyl oximating reagent. This was heated to 60°C for 30 mins resulting in a methoximated standard solution with a concentration of 20ng/ml. This was used to generate of standard curve by dilution in EIA buffer (7.8pg/ml to 1000pg/ml). Next, the cell culture supernatant samples were thawed slowly on ice and 100ml was added

to the same volume of methyl oximating reagent. This solution was heated to 60°C for 30 mins, then centrifuged at 12000g for 5 mins and the supernatant diluted 1:5 in EIA buffer. The required number of microplates were washed in wash buffer prior to use in the assay. 50µl of standard or sample were placed in microplates coated with mouse anti-rabbit monoclonal antibody. To each was added 50µl of PGD_2 conjugate (conjugated to acetylcholinesterase) and 50µl of PGD_2 antibody solution (rabbit antiserum). The plates were covered and incubated overnight at room temperature. After this incubation the wells were aspirated and washed with 200µl wash buffer using a multi-channel pipette. This was repeated to give a total of five washes and after the last wash the microplates were inverted and blotted against clean paper towelling. 200µl of Ellman's reagent was then added to all wells and incubated at room temperature in the dark on a rocking platform for 60 mins. The optical density at 405nm was determined immediately using a Dynatech MR5000 platereader (range required 405-420nm – optimum 412nm). The PGD_2 concentrations were calculated by comparison to the standard curve (linear when plotted on log/lin scale) using BioLinx software.

3.9 Statistics

Statistical analysis was used in two contexts in this work. Firstly, when comparing quantities such as PGE_2 production, statisitical significance was assessed by two-way analysis of variance followed by Dunnett's correction for multiple comparisons with a control. Data on bar charts are expressed as means +/- SEM from three independent experiments unless otherwise stated. $p<0.05$ was taken as the criterion

for a significant difference. Secondly, for comparing half life decay curves for mRNA, statictical significance was assessed by comparing a modelled regression curve to the observed regression curve. By using the sum of the squared deviations about the line an F value can be obtained and tested for significance with the appropriate number of degrees of freeedom. Again, $p<0.05$ was taken as the criterion for a significant difference.

3.10 BUFFERS AND SOLUTIONS

3.10.1 Solutions and reagents for cell culture

Tissue culture reagents were used for cell cultures and all solutions were prepared using sterile MilliQ water.

Phosphate Buffered Saline, pH 7.4 140mM NaCl 2.7mM KCl 1.5mM KH_2HPO_4 8.1mM Na_2HPO_4	**Foetal Bovine Serum** Stored at -20°C in 50ml aliquots (heat-inactivated).
HBSS (pH 7.3) 100ml 10X HBSS 100u/ml penicillin 50u/ml fungizone 100µg/ml streptomycin 50µg/ml gentamicin	**IEC culture medium** 65% Trowell's T8 25% RPMI-1640 10mM HEPES buffer 2mM glutamine 10% FBS Supplemented with penicillin (10 units/ml), streptomycin (10 µg/ml) and fungizone (0.5µg/ml) before use.
Trypsin – EDTA Solution 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA	

HT-29 medium: McCoy's medium 5A	**Caco-2 medium: EMEM (EBSS)**
Supplemented with penicillin (10 units/ml), streptomycin (10 µg/ml) and fungizone (0.5µg/ml) before use.	Supplemented with 2mM Glutamine, 1% Non Essential Amino Acids, penicillin (10 units/ml), streptomycin (10 µg/ml) and fungizone (0.5µg/ml) before use.
Freeze medium – HT-29 cells 10% (v/v) DMSO 40% (v/v) FBS 50% (v/v) HT-29 medium	**Freeze medium – Caco-2 cells** 10% (v/v) DMSO 40% (v/v) FBS 50% (v/v) Caco-2 medium

3.10.2 Solutions and buffers for Nitrite assay

HCl solution	**NaOH solution**
0.62M HCl in MilliQ water.	2.8M NaOH in MilliQ water.

3.10.3 Solutions and buffers for Northern Analysis

3.10.3.1 Required for membrane generation

DEPC- Treated Water or Buffer MilliQ Water or Buffer was treated With 1ml of Diethyl Pyrocarbonate per litre of water and incubated overnight at 37°C. Autoclave (for 20 minutes at 121°C).	**20% (w/v) SDS Solution** 20g SDS diluted in 100ml sterile MilliQ water
75% (v/v) ethanol solution 75 ml absolute ethanol in 25 ml MilliQ water.	**0.5M EDTA stock solution, pH 8** 0.5M EDTA in MilliQ water, adjust pH. Autoclave
3M Sodium acetate, pH 5.2 3M sodium acetate dissolved in 250ml MilliQ water. Adjust pH with 3M glacial acetic acid. DEPC treat and autoclave.	**1mg/ml Ethidium bromide solution** 10mg ethidium bromide in 10ml DEPC-treated water.
20X MOPS running buffer 0.4M 3-[N-morpholino]-propane-sulfonic acid (MOPS) 0.02M EDTA (4 ml of 0.5M stock/100 ml) 0.2M sodium acetate (6.64 ml of 3M stock/100ml) Adjust volume to 100ml with MilliQ water and pH with solid NaOH.	**RNA sample buffer (per sample)** 30µl of: 7µl 36% (w/v) formaldehyde 4µl 20X MOPS running buffer 2µl 1mg/ml ethidium bromide solution 20µl formamide Made up fresh before use.

1X MOPS running buffer 50ml 20X MOPS in 950ml DEPC-treated water.	**20X SSC, pH 7** 3M NaCl 0.3M trisodium citrate dihydrate Milli-Q water, DEPC treat and autoclave
Bromophenol solution 0.025g bromophenol blue 3ml glycerol Adjust volume to 10ml with DEPC-treated water	

3.10.3.2 Required for Digoxigenin labelled probes

Buffer 1 0.1M maleic acid 0.15M NaCl Adjust to pH 7.5 with solid NaOH, DEPC treat and autoclave.	**Blocking stock solution** 10g blocking reagent in 100 ml buffer 1. Microwave to dissolve (do not boil). Autoclave.

Standard Hybridisation solution *For oligonucleotide probes* 0.02% SDS (0.1ml of 20% stock/100ml) 5X SSC (25ml of 20X stock/100ml) 0.1% sarcosyl (0.33ml of 30% stock/100ml) 1% blocking buffer (10ml of 10% stock/100ml) in MilliQ water.	**High SDS Hybridisation solution** *For cDNA probes* 7% SDS (35g of stock/500ml) 5X.SSC (83ml of 30X stock/500ml) 0.1% sarcosyl (5ml of 10% stock/500ml) 2% blocking buffer (100ml of 10% stock/500ml) 50% Formamide (250ml of stock/500ml) 50mM sodium phosphate, pH 7.0 (25ml of 1M stock/500ml) made up to 500ml with MilliQ water and then autoclaved.
1X SSC, 0.1% SDS solution 2.5ml of 20X SSC 2.5ml of 20% SDS stock Adjust volume to 500ml with DEPC-treated water.	**2X SSC, 0.1% SDS solution** 50ml of 20X SSC 2.5ml of 20% SDS stock Adjust volume to 500ml with DEPC-treated water.
Buffer 2 10% (v/v) blocking stock solution in buffer 1.	**Buffer 3, pH 9.5** 0.1M Tris-HCl 0.1M NaCl in sterile Milli-Q water.
Washing buffer 0.3% (v/v) Tween-20 in buffer 1.	**Stripping solution** 0.1% SDS 2.5ml of 20% stock in 500ml MilliQ water

3.10.3.3 Required for [^{32}P] labelled probes

20X SSPE, pH 7.4 3M NaCl 0.2M $NaH_2PO_4.H_20$ 0.02M EDTA pH with NaOH and make up to 1000ml with Milli-Q water and autoclave	**0.5M Na_2HPO_4 (pH 7.2)** 0.5M Na_2HPO_4 in MilliQ water , adjust pH to 7.2 with phosphoric acid
Hybridisation solution 0.001M EDTA 0.2ml of 0.5M stock 0.25M Na_2HPO_4 (pH 7.2) 50ml of 0.5M stock 7% SDS 35ml of 20% stock Make up to 100ml with MilliQ water	**6X SSPE, 0.1% SDS solution** 60ml of 20X SSPE 1ml of 20% SDS stock Adjust volume to 200ml with MilliQ water.
2X SSPE, 0.1% SDS solution 20ml of 20X SSPE 1ml of 20% SDS stock Adjust volume to 200ml with MilliQ water.	**1X SSPE, 0.1% SDS solution** 10ml of 20X SSPE 1ml of 20% SDS stock Adjust volume to 200ml with MilliQ water.
Low Salt solution 0.2M NaCl 4ml of 5M stock 20mM Tris-HCl(pH 7.4) 2ml of 1M stock 1mM EDTA 0.2ml of 0.5M stock Adjust volume to 100ml with MilliQ water	**High Salt solution** 1M NaCl 20ml of 5M stock 20mM Tris-HCl(pH 7.4) 2ml of 1M stock 1mM EDTA 0.2ml of 0.5M stock Adjust volume to 100ml with MilliQ water

3.10.4 Solutions and buffers for SDS-PAGE and Western blotting

Lysis buffer	**Stock solutions**
50mM Tris-HCl pH 7.5 (stock 1M)	1M Tris-HCl pH 8.8 (60.6 g trizma
150mM NaCl (stock 5M)	base/500 ml MilliQ water)
1% (v/v) Nonidet P40 (stock 10% (v/v))	1M Tris-HCl pH 6.8 (60.6 g trizma
10% glycerol	base/500 ml MilliQ water)
5mM EDTA (stock 0.5M, pH 8.0)	Acrylamide/bis acrylamide (30:0.8) (store
1mM sodium orthovanadate	at 4°C)
1mM sodium molybdate	10% (w/v) SDS (100 ml, 10 g SDS +
10mM sodium fluoride	91ml MilliQ water)
40µg/ml phenylmethylsulfonyl fluoride	10% (w/v) ammonium persulphate (1 ml
(PMSF)	aliquots in MilliQ water, store at 4°C)
1µg/ml pepstatin A (stock in methanol)	TEMED
10µg/ml aprotinin	
10µg/ml leupeptin	
10µg/ml soyabean trypsin inhibitor	
MilliQ water	

SDS-PAGE running buffer	**5X SDS-sample buffer**
25mM Trizma base 192mM glycine 0.1% (w/v) SDS MilliQ water, no need to pH, should be above pH 8.3	5% SDS 50% glycerol 200mM Tris-HCl pH 6.8 MilliQ water Bromophenol blue 5% 2-mercaptoethanol (50µl/ml) or 10% 1M DTT (for reducing buffer)
Semi-dry transfer buffer	**Tris-buffered saline (TBS)**
39mM glycine 48mM Trizma base 0.0375% SDS 20% (v/v) methanol	20mM Tris-HCl pH 7.5 150mM NaCl MilliQ water, pH 7.5
Tris-buffered saline (TBSN)	**Ponceau S**
TBS + 0.05% (v/v) NP40	0.1% (w/v) Ponceau S 5% (v/v) acetic acid

Blocking buffer	**Coomassie blue stain**
For COX antibodies: 5% (w/v) non-fat powdered milk (Marvel) in TBS 0.05% azide For other antibodies: 5% BSA (w/v) 1% ovalbumin (w/v) 0.05% azide	0.25% (w/v) Coomassie blue 45.4% (v/v) methanol 9.2% (v/v) glacial acetic acid Milli-Q water
Stripping buffer	**Destain solution**
62.5 mM Tris-HCl, pH 6.8 2% (w/v) SDS 100mM 2-mercaptoethanol MilliQ water	45.4% (v/v) methanol 9.2% (v/v) glacial acetic acid MilliQ water

3.10.4.1 Recipes for various percentage gels

Resolving gel – 5 ml is sufficient for 1 mini gel

Stacking gel* – 1.5 ml is sufficient for 1 mini gel

Final % gel	**5%***	**7.5%**	**10%**
Range (kDa)		***70-200***	***20-100***
Acrylamide	1.67	3.75	5
MilliQ H_2O	6	5.6	4.35
1M Tris pH 8.8		5.6	5.6
1M Tris pH 6.8	1.25		
10% SDS	0.15	0.25	0.25
Total (ml)	9.07	15.2	15.2

Plus 50µl 10% APS and 20µl TEMED

3.10.4.2 Antibodies used for immunoblotting

Primary antibody	**Molecular Weight (kDa)**	**Secondary antibody**
Cyclo-oxygenase 1	68-74	Anti-goat HRP
Cyclo-oxygenase 2	68-74	Anti-goat HRP
Pan PKB	60	Anti-rabbit HRP
Phospho^{Ser473} PKB	60	Anti-rabbit HRP
Pan p38	43	Anti-rabbit HRP
Phospho$^{Thr180/Tyr182}$ p38	43	Anti-rabbit HRP
Pan ERK1/2	42/44	Anti-rabbit HRP
Phospho$^{Thr202/Tyr204}$ ERK1/2	42/44	Anti-rabbit HRP
Pan JNK	46/54	Anti-rabbit HRP
Phospho$^{Thr183/Tyr185}$ JNK	46/54	Anti-rabbit HRP
Pan GSK-3α/β	46/51	Anti-rabbit HRP
Phospho$^{Ser21/9}$ GSK-3α/β	51α 46β	Anti-rabbit HRP
Phospho^{Thr71} ATF-2 (ATF-2 GST fusion Protein)	70 39	Anti-rabbit HRP

3.10.5 Solutions and buffers measuring phosphatidylinositol levels

Phosphate buffer	**Phosphate free medium**
1.25M *di*-ammoniumhydrogen orthophosphate, $(NH_4)_2HPO_4$ Milli-Q water pH adjusted to 3.8 using orthophosphoric acid	Add powdered DMEM with L-Glutamine and 4500mg glucose to 900ml MilliQ water. Add 49.3ml (7.5% (w/v)) sodium bicarbonate, 20ml of IM HEPES (20mM). Adjust pH to 7.2 and adjust volume to 1l. Filter sterilise and store at 4°C

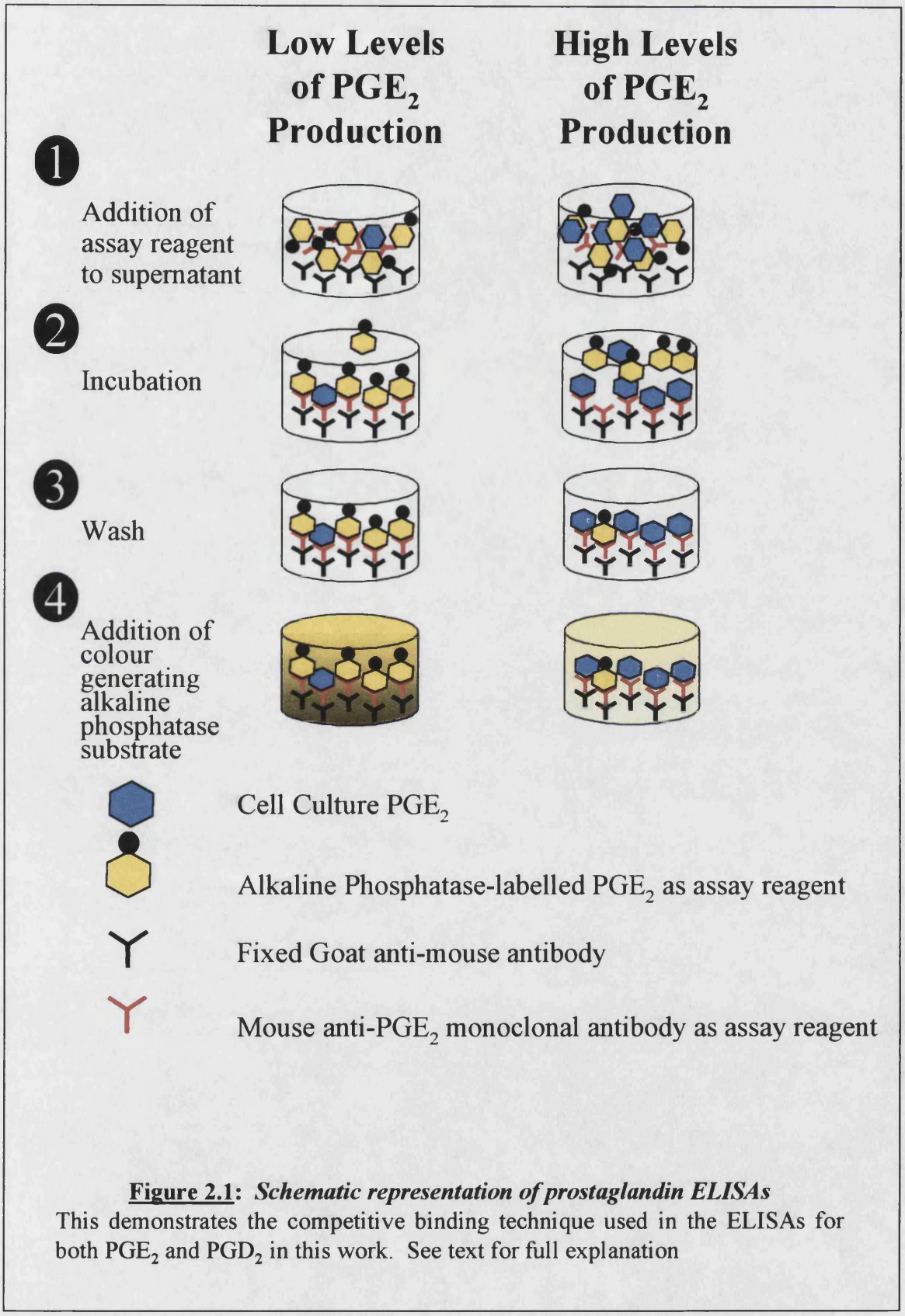


Figure 2.1: ***Schematic representation of prostaglandin ELISAs***
This demonstrates the competitive binding technique used in the ELISAs for both PGE_2 and PGD_2 in this work. See text for full explanation

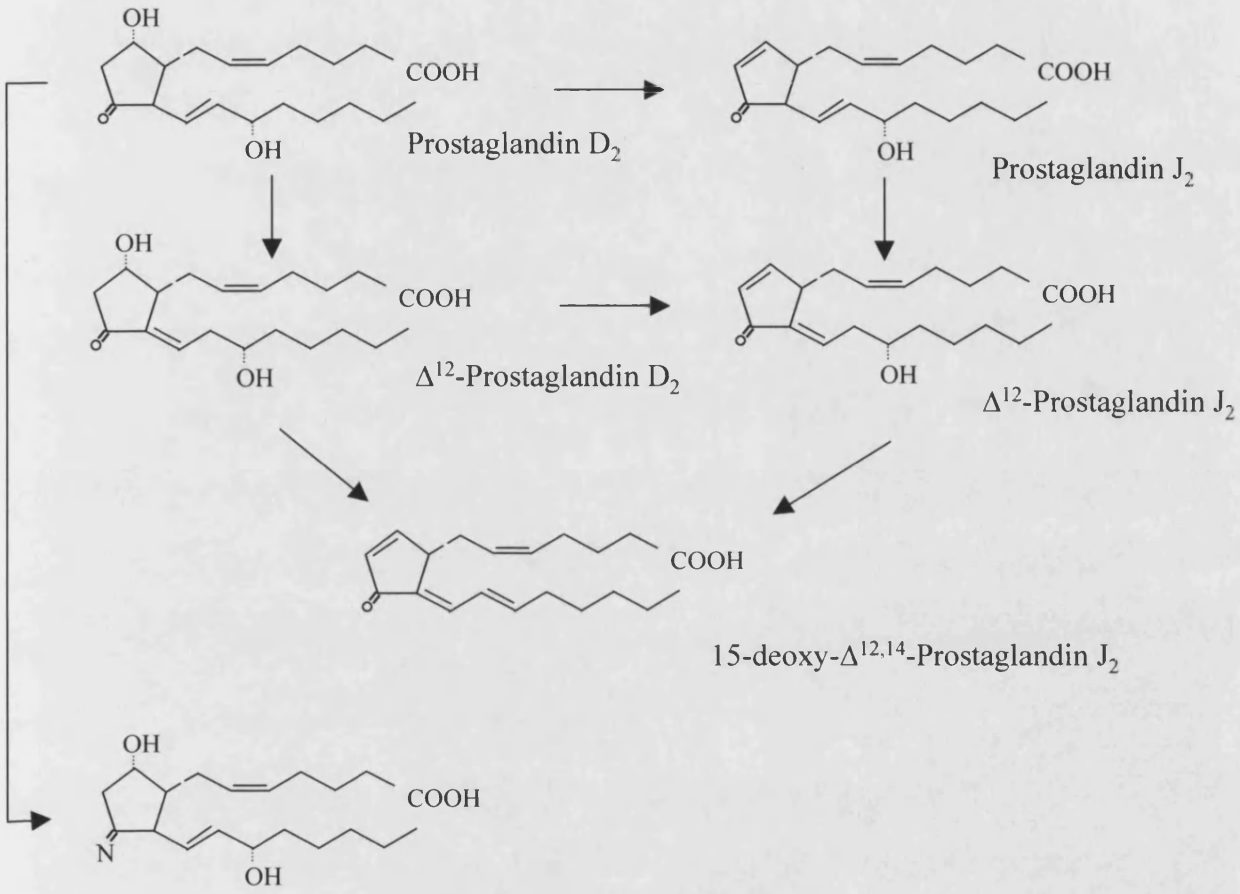


Figure 2.2: ***Schematic representation of PGD_2 derivatives***

This demonstrates the metabolism of PGD_2 to both the J series of cyclopentenone prostaglandins, as well as the conversion to the stable methoximated derivative

4 Results - Induction of COX-2

4.1 Introduction

COX-2 is known to be an inducible enzyme which has been shown to be induced by a variety of agents in a number of systems. With respect to the intestinal epithelium, this work concentrated on whether COX-2 was induced by pro-inflammatory mediators known to play a role in intestinal pathophysiology. In order to investigate this hypothesis, techniques needed to be developed to assess COX-2 induction at the mRNA, protein and product level. Furthermore, the cellular models for the intestinal epithelium needed to be chosen and the results interpreted in the context of the model used.

Having established the basic properties of COX-2 induction in the model used, these results could be used as a foundation for further work. In particular, although COX-2 is known to be highly regulated, it is one link in a long path from arachidonic acid liberation to prostanoid generation and metabolism. It has been demonstrated in other systems that COX-2 products can feed back and regulate COX-2 expression and activity. The existence of any such feedback loops is important in the interpretation of all experiments on COX-2 regulation.

Finally 5-ASA, which is known to have therapeutic value in IBD and have a multiplicity of differing actions, was used to see whether it altered COX-2 expression and activity.

4.2 Results

4.2.1 Development of COX-2 Probe

4.2.1.1 Digoxigenin labelled COX probe

In order to investigate COX expression at the mRNA level an assay for COX mRNA had to be developed. Northern blot analysis was chosen over PCR based methods because it allows a direct measurement of the total amount of a specific mRNA and avoids the assumptions of reverse transcription seen with PCR. A probe, suitable for northern analysis was therefore required, and initial attempts used the Digoxigenin labelling of COX-1 and COX-2 cDNA as described in Methods. COX-2 cDNA could be labelled reliably with Digoxigenin and this was shown using a dot blot to compare the Digoxigenin dependent signal to control labelled DNA (Figure 4.1A). However, the probe proved very sensitive to conditions in a manner which was not always predictable. Figure 4.1B and 4.1C show the effect of probe concentration and temperature of hybridisation on mock blot development ie: membranes without any mRNA present. As probe concentration increased, or temperature decreased, the intensity of the background signal rose to unacceptable levels. Some successful probing was carried out allowing interpretation of COX-2 mRNA expression but, in view of the high and unpredictable background, a new method of labelling COX-2 cDNA was sought.

4.2.1.2 [^{32}P] labelled COX-2 probe

The technique of labelling COX-2 cDNA with [^{32}P] shares many methodological similarities as that for Digoxigenin labelling. However the predictability of the results, the low levels of background and the resulting increased sensitivity meant that this proved to be a superior method. Figure 4.1D shows a comparison between duplicate membranes probed with either Digoxigenin or [^{32}P] labelled COX-2 probes with the latter much clearer. As a result all COX-2 northern blots shown are probed with [^{32}P] labelled COX-2 cDNA. Figure 4.1D also compares the methods of demonstrating equal loading: either stripping and reprobing for the housekeeping gene β-Actin, or showing the photograph of the ethidium stained blot where the 18S and 28S ribosomal RNA bands can be visualised. There was no difference seen between these two methods during this work and they are both used throughout.

4.2.2 Induction of COX-2

It is commonly reported that COX-2 is commonly inducible in other cell lines by stimulation with a variety of pro-inflammatory cytokines, bacterial products, tumour promoters and other mitogens. Therefore the pro-inflammatory Th1 cytokines IL-1 and TNFα were initially chosen in an attempt to induce COX-2 in intestinal epithelial cells.

4.2.2.1 COX-2 mRNA and protein induction in HT-29 Cells

HT-29 intestinal epithelial cells were stimulated over a twenty four hour time course with TNFα or IL-1α and mRNA was isolated for northern blot analysis for COX-2

(Figure 4.2A). Both cytokines caused COX-2 induction although that by TNFα was both more intense, appeared quicker and was more prolonged. Whereas the response to IL-1α was a peak of COX-2 mRNA at two hours, the response to TNFα was more complex. TNFα also caused an initial peak in COX-2 mRNA at two hours but, after a decline in intensity at four hours, there was a second peak in COX-2 expression at twelve hours.

In view of both IL-1α and TNFα both inducing COX-2 mRNA with an early peak at two hours, this time point was chosen to perform a dose response. HT-29 cells were stimulated with increasing concentrations of either TNFα (0-100ng/ml) or IL-1α (0-30ng/ml) for two hours and mRNA was isolated and analysed by northern blot. Both cytokines showed a concentration dependent response with visible induction even at low concentrations (Figure 4.2B). Subsequent experiments therefore used TNFα at 100ng/ml and IL-1α at 10ng/ml as proven potent inducers of COX-2 in HT-29 cells.

As there are two isoforms of IL-1, IL-1α and IL-1β, the response of HT-29 cells to IL-1β stimulation (0-10ng/ml) for two hours was also assessed with regard to COX-2 induction (Figure 4.2C). IL-1β proved to induce COX-2 mRNA and was more potent than IL-1α but not TNFα. It also demonstrated a plateau effect with no increase in induced signal above 1ng/ml.

Finally, regarding COX-2 mRNA induction in HT-29 cells, LPS (10μg/ml) and Interferon-γ (IFNγ) (300u/ml) failed to induce COX-2 mRNA over a twenty four hour period (Figure 4.2D). This figure also demonstrates the very low levels of basal

COX-2 mRNA seen, representing the small amount of constitutive expression in this colorectal carcinoma derived cell line.

In light of TNFα and IL-1α inducing COX-2 mRNA in HT-29 cells, western blot analysis was used to assess whether the same stimulation also caused induction of COX-2 protein. HT-29 cells were stimulated over a six hour time course with TNFα (100ng/ml) and protein was isolated, analysed by western blotting and probed for COX-2 using a specific anti-COX-2 antibody (Figure 4.3A). A time dependent induction of COX-2 protein is seen from three hours and a continuing rise at six hours. This lags behind the induction of COX-2 mRNA as would be expected in order to allow for protein translation. It can also be seen that stimulation with TNFα over the same time period has no effect on COX-1 protein expression, which is constitutively expressed at low levels throughout the time course (Figure 4.3A). Using six hours as an early point giving clear increases in COX-2 protein, TNFα was compared to IL-1α (10ng/ml) in the same cells (Figure 4.3B). IL-1α is seen to induce COX-2 protein but this induction is weaker in comparison to that seen with TNFα.

4.2.2.2 COX-2 mRNA and protein induction in Caco-2 Cells

The Caco-2 cell line was chosen as a second model of intestinal epithelial cells. To investigate the induction of COX-2 expression in this cell line, cells were stimulated with TNFα (100ng/ml), LPS (10μg/ml), IFNγ (300u/ml) or IL-1α (10ng/ml) over a twenty four hour time course. mRNA was isolated and probed for COX-2 using northern blot analysis (Figure 4.4A). As can be seen, IL-1α was the only agent

capable of inducing COX-2 mRNA expression in Caco-2 cells. As with HT-29 cells this induction peaks at two hours.

Subsequent to the finding of induction of COX-2 mRNA in Caco-2 cells, IL-1α was used to stimulate cells over a six hour time course with protein being isolated for western blot analysis (Figure 4.4B). This showed that IL-1α caused a concomitant time dependent induction of COX-2 protein in Caco-2 cells although this was not of great magnitude.

4.2.2.3 Induction of Prostaglandin production as a marker of COX-2 activity

Downstream of COX activity, specific isomerases determine production of the individual prostaglandins. PGE_2 is the most abundant prostaglandin product in the colon (Boughton-Smith *et al.*, 1983) and appeared to be a good product to assay as a potential downstream marker of COX-2 functional activity. HT-29 cells were stimulated in triplicate with TNFα over a 72 hour time course with supernatants being assayed for PGE_2 using a specific ELISA (Figure 4.5A). This showed that TNFα induced a time dependent increase in PGE_2 production which was still increasing at 72 hours. There is some evidence that serum deprivation may cause physiological changes in cellular responses in its own right (Battu *et al.*, 1998). In light of this, and although the appropriate controls did not support this with respect to PGE_2 production, PGE_2 assays tended to be carried out over 24-48 hours.

Next it was investigated whether TNFα induced PGE_2 production was concentration dependent, as has already been demonstrated with COX-2 mRNA production. Also,

if it is assumed that inducible PGE_2 production in this system is indeed COX-2 dependent, it should be inhibited by a specific COX-2 inhibitor. HT-29 cells were stimulated in triplicate for twenty four hours with increasing concentrations of TNFα (10 and 100ng/ml) with the higher concentration also being used to stimulate cells which had been pre-treated with the specific COX-2 inhibitor NS-398 for one hour at 1μM (Figure 4.5B). The results showed that TNFα induction of PGE_2 production was indeed both concentration dependent and COX-2 dependent.

Finally the possibility of PGE_2 induction by IL-1α both in HT-29 cell and in the Caco-2 cell line was explored. Caco-2 and HT-29 cells were stimulated in triplicate with IL-1α (10ng/ml) for 72 hours whilst HT-29 cells were incubated with TNFα as a positive control. The prolonged time period was chosen in the hope of enhancing the sensitivity of the assay. IL-1α did not stimulate PGE_2 production in either cell line despite its proven ability to induce COX-2 expression (Figure 4.5C).

It is known that downstream of COX-2 there are a variety of differing COX-2 dependent products and it has been proposed that these may have opposing actions and therefore be regulated differently (Gilroy *et al.*, 1999). The cyclopentenone prostaglandins are believed to have differing actions to PGE_2 and, although these are unstable, a stable derivative of the PGD_2 can be assayed using an ELISA technique. HT-29 cells were stimulated over twenty four or forty eight hours with a variety of possible inducing agents: TNFα (100ng/ml), IL-1α (10ng/ml), IFNγ (300u/ml) both alone and in combination with TNFα or IL-1α, and the Th2 cytokines IL-4 and IL-13 (both 30ng/ml). Supernatants were collected and assayed for the stable methoximated derivative of PGD_2 as a marker of PGD_2 production (Figure 4.6). The

assay, which worked well with a standard curve acting as a positive internal control, revealed that none of these stimuli induced methoximated PGD_2, and by implication PGD_2 production, in HT-29 cells at a level to be assayed using this ELISA method.

4.2.2.4 Expression of COX-2 in isolated primary intestinal epithelial cells

In the hope of supporting the above findings in colonic epithelial cell lines, attempts were made to investigate COX-2 expression in freshly isolated human colonic epithelial cells. It is known that these cells do express COX-2 when involved in colonic tumours or in colonic inflammation (Eberhart *et al.*, 1994; Singer *et al.*, 1998). It is also reported in the literature that they have receptors to IL-1 although not TNFα (Panja *et al.*, 1998), and a functional response to IL-1β has been demonstrated (Jobin *et al.*, 1998).

Primary intestinal epithelial cells (IECs) were isolated from fresh colonic resection specimens and aliquots of 10^7 cells were either stimulated for two hours with IL-1β (10ng/ml) or left unstimulated as a control. mRNA was then isolated and analysed by northern blot using HT-29 cells stimulated by TNFα as a positive control (Figure 4.7A). There was no visible induction of COX-2 mRNA in these fresh IECs as compared to that seen in HT-29 cells. To investigate this further, IECs were similarly stimulated for sixteen hours with IL-1β (10ng/ml) and protein was isolated for western analysis and probing for COX-2 (Figure 4.7B). Again there was no visible induction of COX-2 protein by IL-1β although there appeared to be a small amount present constitutively.

4.2.3 Regulation of COX-2 by its Products

4.2.3.1 PGE_2

It has been demonstrated above that TNFα induction of COX-2 results in a downstream induction of COX-2 dependent PGE_2 production. PGE_2 is the greatest prostaglandin product produced in the colon and the question was raised as to whether there was any positive or negative feedback on COX-2 mRNA or protein expression. HT-29 cells were stimulated in the presence or absence of TNFα (100ng/ml) and in turn in the presence or absence of pre-treatment with increasing concentrations of PGE_2 (0-5000pg/ml) for either two hours for mRNA isolation or six hours for isolation of protein. COX-2 mRNA, as well as COX-2 and COX-1 protein levels, were analysed by northern and western blotting respectively. The concentrations of PGE_2 chosen were based on the amounts produced in this system as quantified by ELISA. The presence of PGE_2 caused a concentration dependent inhibition of COX-2 mRNA and protein production in these HT-29 cells and this was best seen at the highest concentrations of 2500-5000pg/ml (Figure 4.8A and 4.8B). PGE_2 on its own had no effect on COX-2 expression even at high concentrations. Also, the protein expression of the constitutive isoform of COX, COX-1, was unaltered either by TNFα or by PGE_2 (Figure 4.8B lower panel).

4.2.3.2 PPARγ agonists

As mentioned earlier with respect to prostaglandin induction, differing COX-2 dependent products have been shown to have differing and indeed opposing actions. The cyclopentenone prostaglandins are known to mediate their actions by binding to

the nuclear hormone receptor PPARγ. As well as cyclopentenone prostaglandins such 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 (PGJ_2), drugs are now available, such as ciglitazone - a member of the thiazolidinedione family, which can act as PPARγ agonists. PGJ_2 and ciglitazone were used to investigate the actions of PPARγ agonists on cytokine induced COX-2 expression in HT-29 intestinal epithelial cells.

Cells were stimulated with TNFα in the presence or absence of pre-treatment with PGJ_2 or ciglitazone (one hour at 10μM) and mRNA was isolated at two hours for northern blot analysis and COX-2 probing (Figure 4.9A). Neither PPARγ agonist was seen to have a significant effect on COX-2 expression at the mRNA level. To see whether the same could be said for COX-2 protein expression, HT-29 cells were stimulated for six hours as above and protein isolated for western blot analysis and COX-2 probing (Figure 4.9B). This revealed that both PPARγ agonists caused an added up-regulation in TNFα induced COX-2 protein expression.

Finally, experiments were carried out to assess whether this up-regulation of induced COX-2 protein by PPARγ agonists was reflected in downstream PGE_2 production. HT-29 cells were stimulated with TNFα (100ng/ml) and IFNγ (300u/ml) to provide a potent induction of PGE_2 production as further discussed in Chapter 5. This was carried out in the presence or absence of either PGJ_2 or ciglitazone for twenty four hours and supernatants were collected and analysed for PGE_2 by ELISA (Figure 4.9C). The marked induction of PGE_2 by TNFα and IFNγ was inhibited by the PPARγ agonists and this effect was significant and far more marked for PGJ_2 (≈90% inhibition - $p<0.01$) than for ciglitazone (≈25% inhibition). For PGE_2 production, as

well as COX-2 mRNA and protein expression, neither PPARγ agonist had any effect in isolation.

4.2.4 Regulation of COX-2 by 5-ASA

5-ASA is a widely used therapeutic agent in IBD that has been shown to have a variety of actions (Greenfield *et al.*, 1993). Although it has been shown to alter prostaglandin production in the gastrointestinal tract (Hawkey and Truelove, 1983), this work was carried out prior to the description of an inducible isoform of COX. The action of 5-ASA on COX-2 expression and activity in HT-29 cells was therefore specifically investigated. Cells were stimulated with TNFα (100ng/ml) in the presence or absence of pre-treatment with increasing, therapeutically relevant, concentrations of 5-ASA (10-1000μM for one hour), or equivalent amounts of its dilute HCl vehicle. At two hours mRNA was isolated and subsequently analysed by northern blot and probed for COX-2. This showed a minimal inhibitory effect of 5-ASA, only at the highest concentration of 1000μM compared to its vehicle, on COX-2 mRNA expression (Figure 4.10A).

To see whether this relative lack of regulation of COX-2 by 5-ASA extended beyond the mRNA level, TNFα induced PGE_2 production was assessed in the presence of 5-ASA. HT-29 cells were stimulated for 72 hours with TNFα as above in the presence or absence of 5-ASA or its HCl vehicle. Supernatants were collected and the PGE_2 content was quantified with ELISA (Figure 4.10B). This showed that 5-ASA, whilst having no effect in isolation, caused a significant concentration dependent inhibition

of TNFα induced PGE_2 production. This inhibition was complete at 1000μM and significantly greater than the inhibition caused by the vehicle alone (≈50% at equivalent HCl to 1000μM 5-ASA). These results gave an IC_{50} for 5-ASA on TNFα induced PGE_2 production of 119μM.

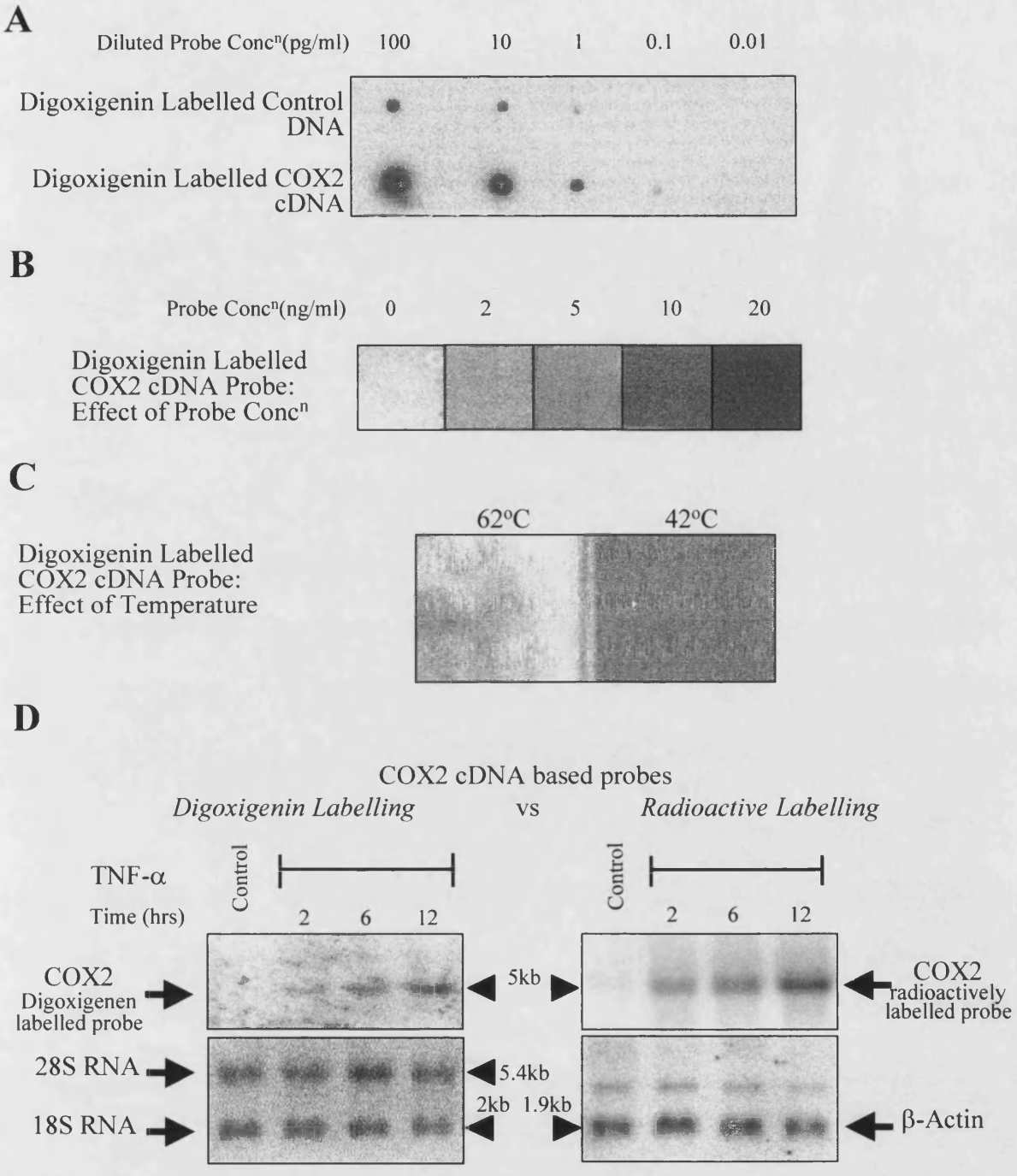


Figure 4.1: ***Development of COX2 probe for northern analysis.***

A. Dot blot of Digoxigenin labelled COX2 cDNA at a variety of predicted concentrations (100-0.01pg/ml) compared to a control Digoxigenin labelled standard DNA. **B**. Five mock blots each carried out using different concentrations of Digoxigenin labelled COX2 probe (0-20ng/ml) and developed at the same time in the same way. **C**. Two mock blots carried at two different hybridisation temperatures (42°C and 62°C) with the same concentration of Digoxigenin labelled COX2 probe (25ng/ml). **D**. Comparison of Digoxigenin labelled versus [^{32}P] labelled COX2 cDNA probes on duplicate membranes (upper panels). HT-29 cells were stimulated with TNFα (100ng/ml) over a 12 hour time course. The lower panels demonstrate equal loading with a photo of the ethidium stained 18S and 28S ribosomal RNA bands on the left; and the membrane stripped and reprobed for β-actin on the right.

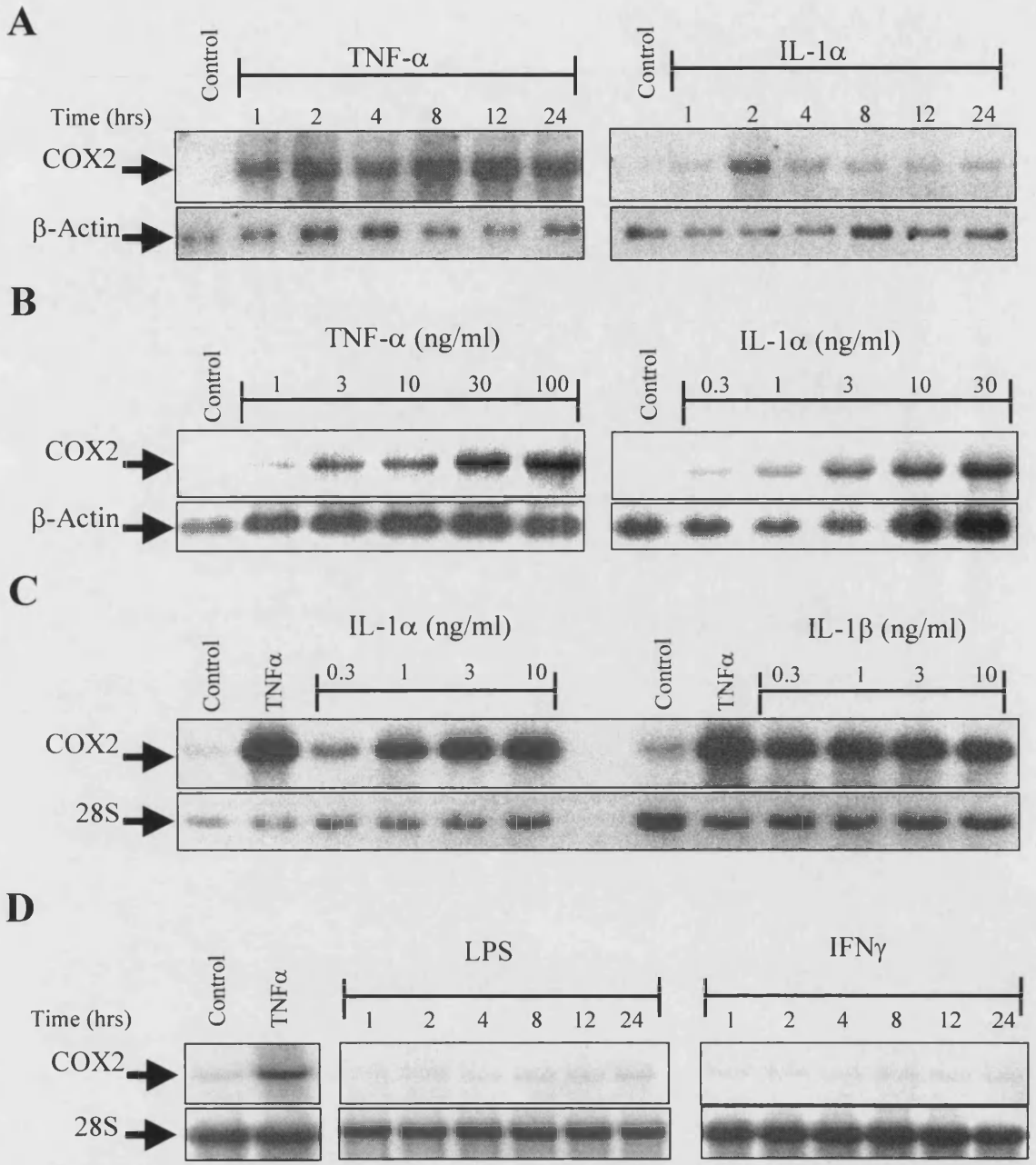


Figure 4.2: ***Induction of COX2 mRNA in HT-29 cells.***

A to D. Northern analyses of mRNA isolated from HT-29 cells and probed for COX2. **A**. Cells were stimulated with TNFα (100ng/ml - left) or IL-1α (10ng/ml - right) over a 24 hour time course. The membranes were stripped and reprobed for β-actin to demonstrate loading. **B**. Cells were stimulated for two hours with increasing concentrations of TNFα (0-100ng/ml - left) or IL-1α (0-30ng/ml - right). The membranes were stripped and reprobed for β-actin to demonstrate loading. **C**. Cells were stimulated for two hours with increasing concentrations of IL-1α (0-10ng/ml - left) or IL-1β (0-10ng/ml - right) and compared to unstimulated control and TNFα (100ng/ml). The 28S band is shown to demonstrate loading. **D**. Cells were stimulated over a 24 hour time course with LPS (10μg/ml) or IFNγ (300u/ml) and compared to unstimulated control and TNFα (100ng/ml). The 28S band is shown to demonstrate loading. Blots were from single experiments but are representative of at least three others.

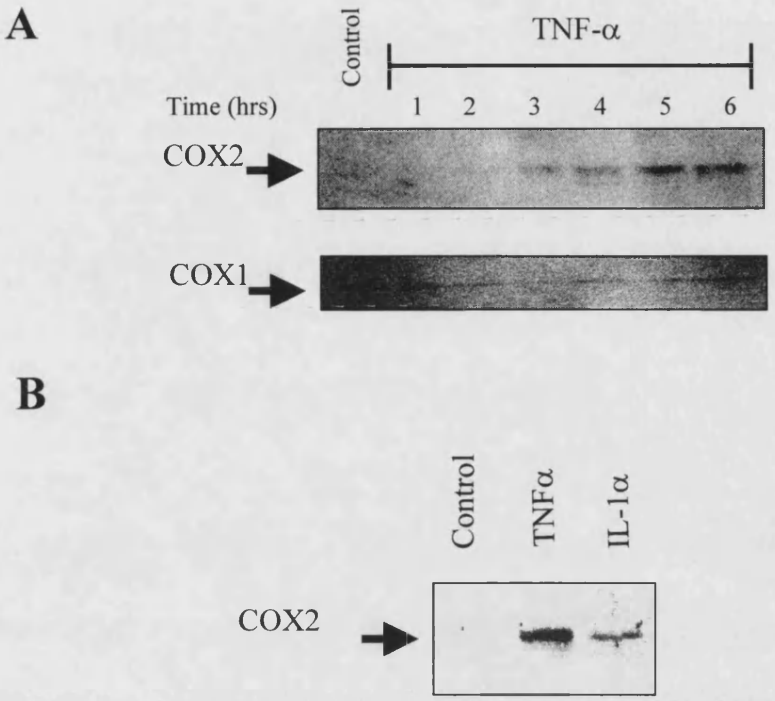


Figure 4.3: ***Induction of COX2 protein in HT-29 cells.***

A. Western analysis of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) over a six hour time course and probed with a specific anti-COX2 antibody (upper panel) or a specific COX1 antibody (lower panel). **B**. Western analysis of protein isolated from HT-29 cells comparing stimulation for six hours with either TNFα (100ng/ml) or IL-1α (10ng/ml) and probed with a specific anti-COX2 antibody . Blots were from single experiments but are representative of three others.

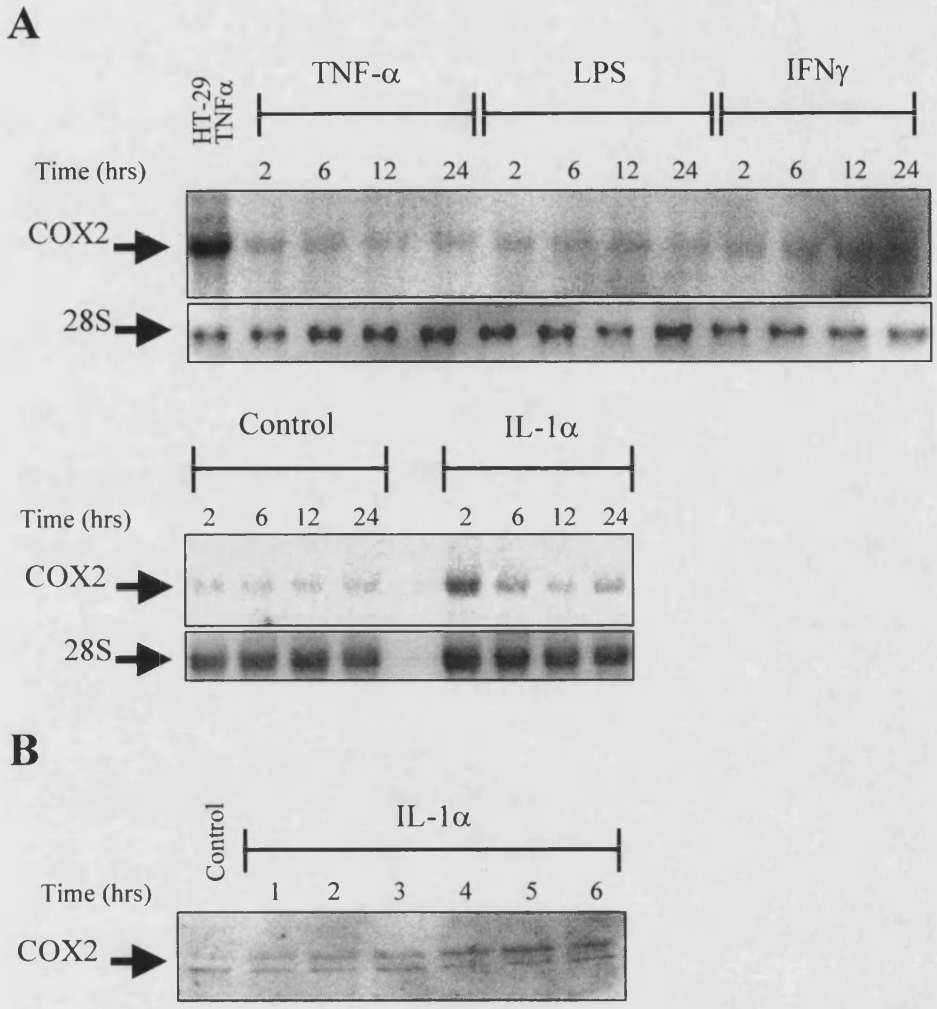


Figure 4.4: ***Induction of COX2 mRNA and protein in Caco-2 cells.***
A. Northern analysis of mRNA isolated from Caco-2 cells and probed for COX2. Cells were stimulated with TNFα (100ng/ml), LPS (10μg/ml), IFNγ (300u/ml) or IL-1α (10ng/ml) over a 24 hour time course and compared to unstimulated cells. The 28S band is shown to demonstrate loading. **B.** Western analysis of protein isolated from cells stimulated with IL-1α (10ng/ml) over a six hour time course. Blots were from single experiments but are representative of at least three others.

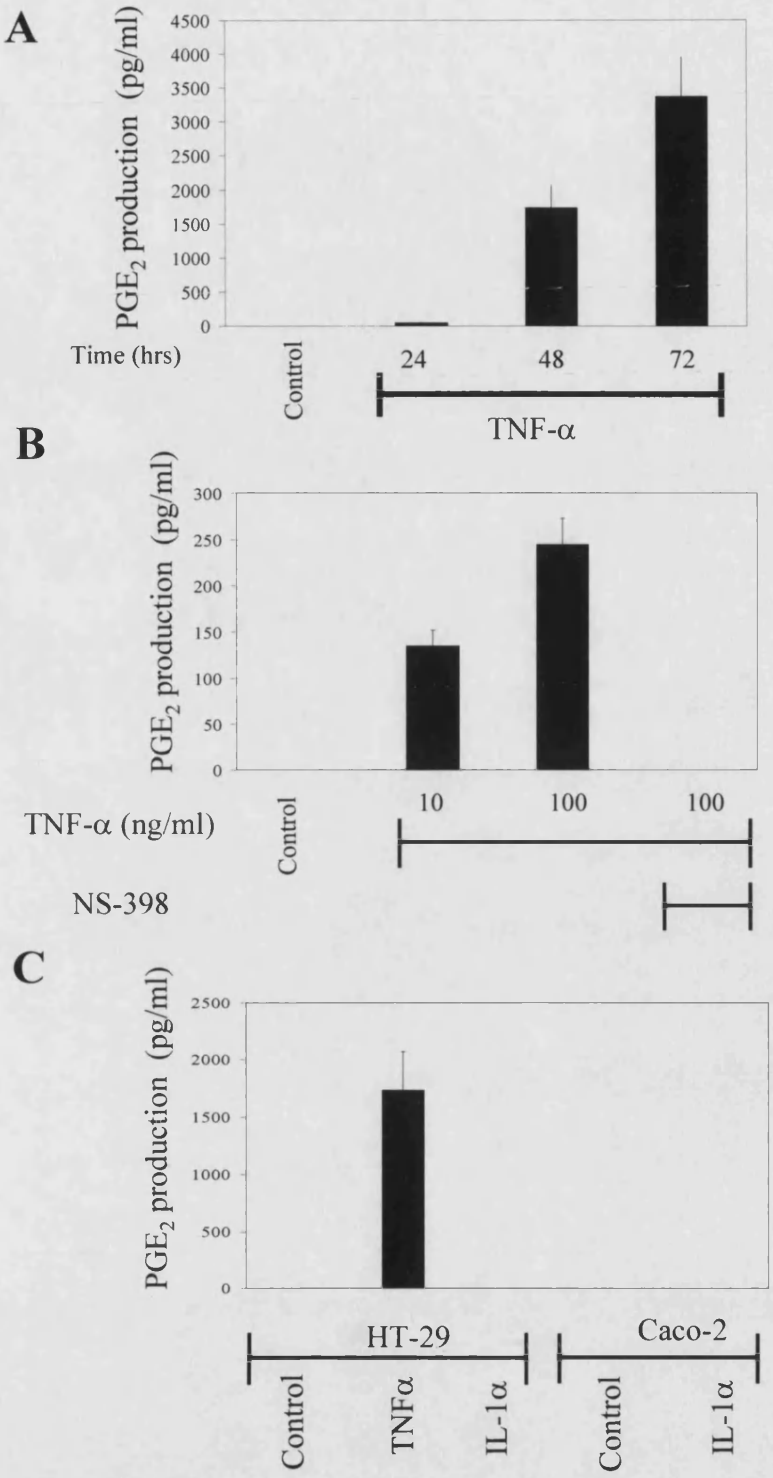
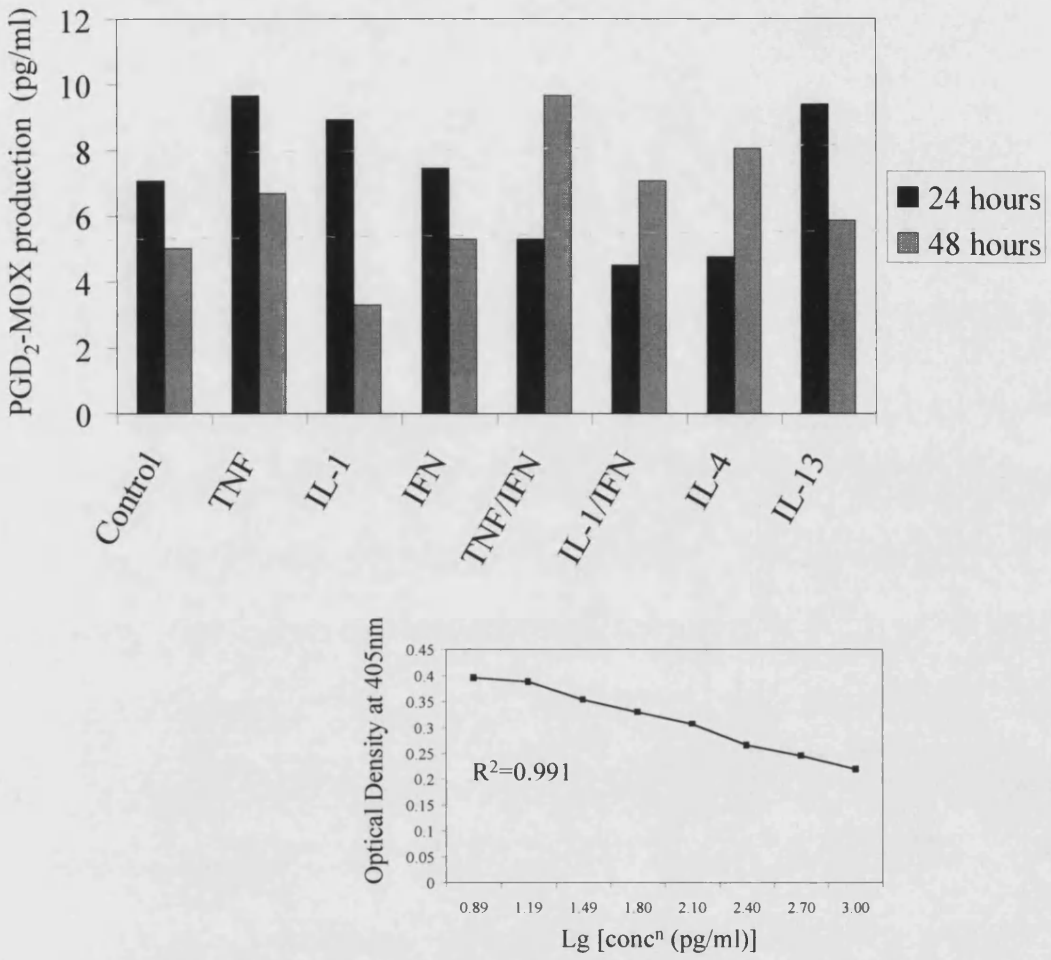


Figure 4.5: ***Stimulation of PGE_2 production in intestinal epithelial cells.***

A. Bar chart showing PGE_2 production by HT-29 cells stimulated with TNFα (100ng/ml) over a 72 hour time course, prior to removal of the cell culture supernatant for PGE_2 assay by ELISA. **B**. Bar chart showing PGE_2 production by HT-29 cells stimulated with increasing concentrations of TNFα (0-100ng/ml) for 24 hours. This is compared to stimulation with TNFα (100ng/ml) in the presence of pre-treatment with the specific COX2 inhibitor NS-398 (1μM for one hour). **C**. Bar chart representing PGE_2 production in both HT-29 cells and Caco-2 cells. Cells were stimulated for 72 hours with TNFα (100ng/ml - HT-29 cells only) or IL-1α (10ng/ml - both Caco-2 and HT-29 cells) and compared with unstimulated cells. Cells were stimulated in triplicate and the results represent at least two independent experiments.



PGD_2-MOX EIA Standard Curve

Figure 4.6: ***Methoximated PGD_2 production in HT-29 cells.***

Bar chart showing production of the stable PGD_2 derivative (PGD_2-MOX) by HT-29 cells stimulated for 24 or 48 hours with a variety of stimuli: TNFα (100ng/ml), IL-1α (10ng/ml), IFNγ (300u/ml), IFNγ combined with TNFα or IL-1α, IL-4 and IL-13 (both 30ng/ml). The standard curve for the assay is shown beneath to demonstrate the internal positive control.

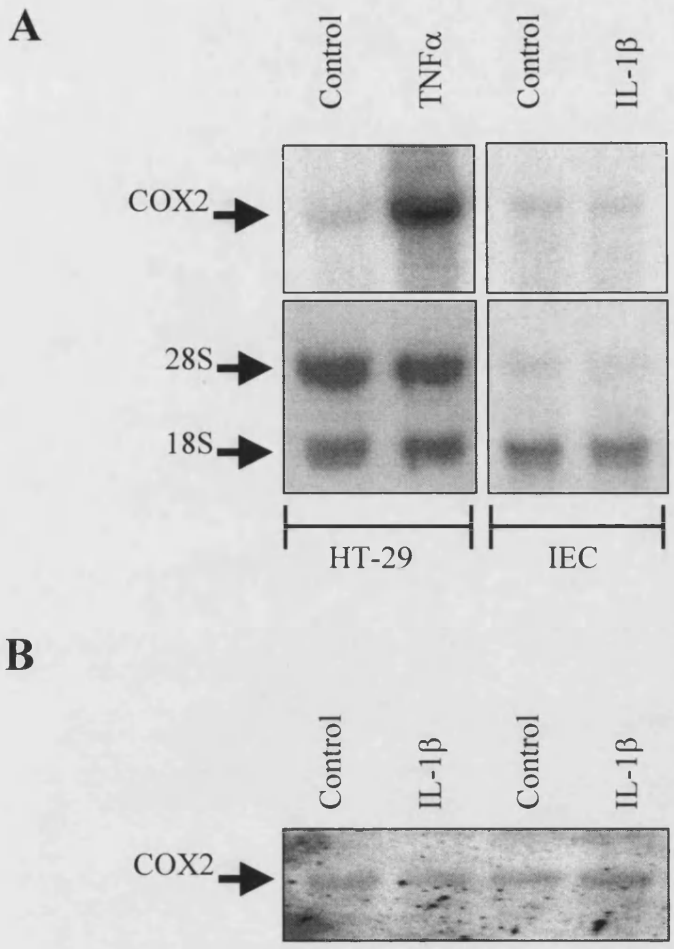


Figure 4.7: ***COX2 expression in primary intestinal epithelial cells***.
A. Northern analysis of mRNA comparing HT-29 cells, both stimulated for two hours with TNFα (100ng/ml) or left unstimulated, with 10^7 primary intestinal epithelial cells stimulated with IL-1β (10ng/ml) or left unstimulated, and probed for COX2. Both the 18S and 28S bands are shown to demonstrate loading. **B**. Western analysis of protein isolated from primary intestinal epithelial cells stimulated for 16 hours with IL-1β (10ng/ml) compared to unstimulated control, and probed for COX2. Blots are from single experiments but are representative of at least one other.

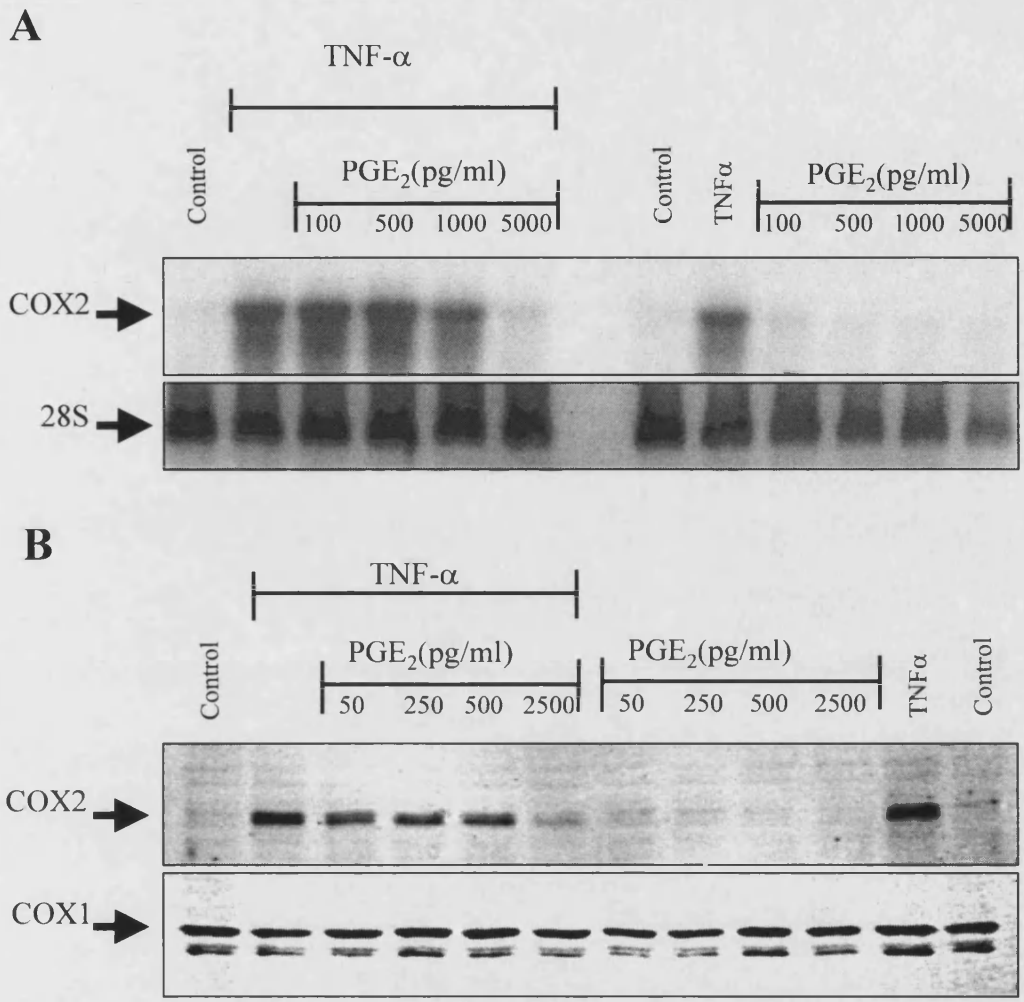


Figure 4.8: ***Regulation of induced COX2 expression by PGE_2.***

A. Northern analysis of mRNA isolated from HT-29 cells pre-treated with increasing concentrations of PGE_2 (0-5000pg/ml for one hour prior) in the presence or absence of TNFα (100ng/ml for two hours) as the stimulating cytokine and probed for COX2. The 28S band is shown to demonstrate loading. **B**. Western analysis of protein isolated from HT-29 cells pre-treated with increasing concentrations of PGE_2 (0-2500pg/ml for one hour) in the presence or absence of TNFα (100ng/ml for six hours) as the stimulating cytokine and probed for COX2. The membrane was stripped and reprobed with a specific antibody against COX1 showing equal loading. Some of these results were obtained supervising Miss K.M. Patel in her final year project.

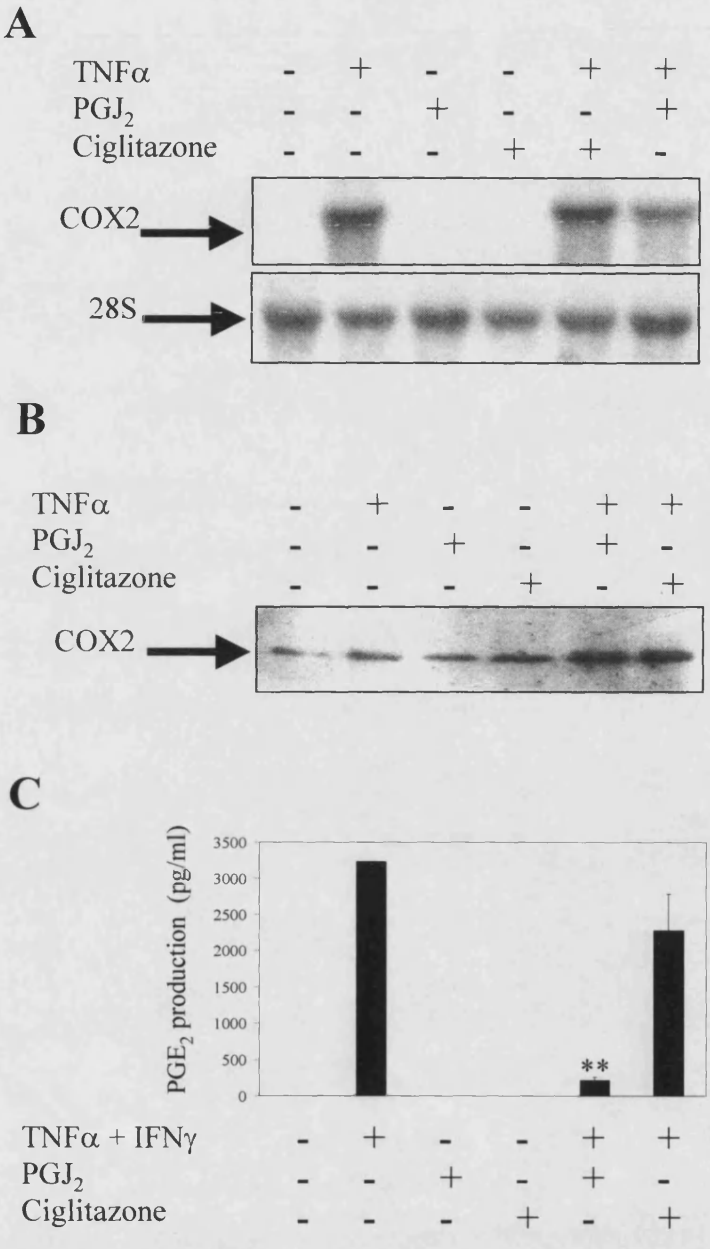


Figure 4.9: ***Regulation of COX2 expression by PPARγ agonists.***

A. Northern analysis of mRNA isolated from HT-29 cells stimulated with TNFα (100ng/ml) for two hours in the presence or absence of pre-treatment with the PPARγ agonists PGJ_2 or ciglitazone (both at 10μM for one hour) and probed for COX2. The 28S band is shown to demonstrate loading. **B.** Western analysis of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) for six hours in the presence or absence of pre-treatment with PGJ_2 or ciglitazone (both 10μM for one hour) and probed with a specific antibody against COX2. **C.** Bar chart representing PGE_2 production by HT-29 cells stimulated by TNFα (100ng/ml) and IFNγ (300u/ml) for 24 hours in the presence or absence of pre-treatment with PGJ_2 or ciglitazone (both 10μM for one hour). ** represents a significant inhibition at $p<0.01$. Blots were from single experiments but are representative of one other. Some of these results were obtained supervising Mr D.G. Cronshaw in his final year project.

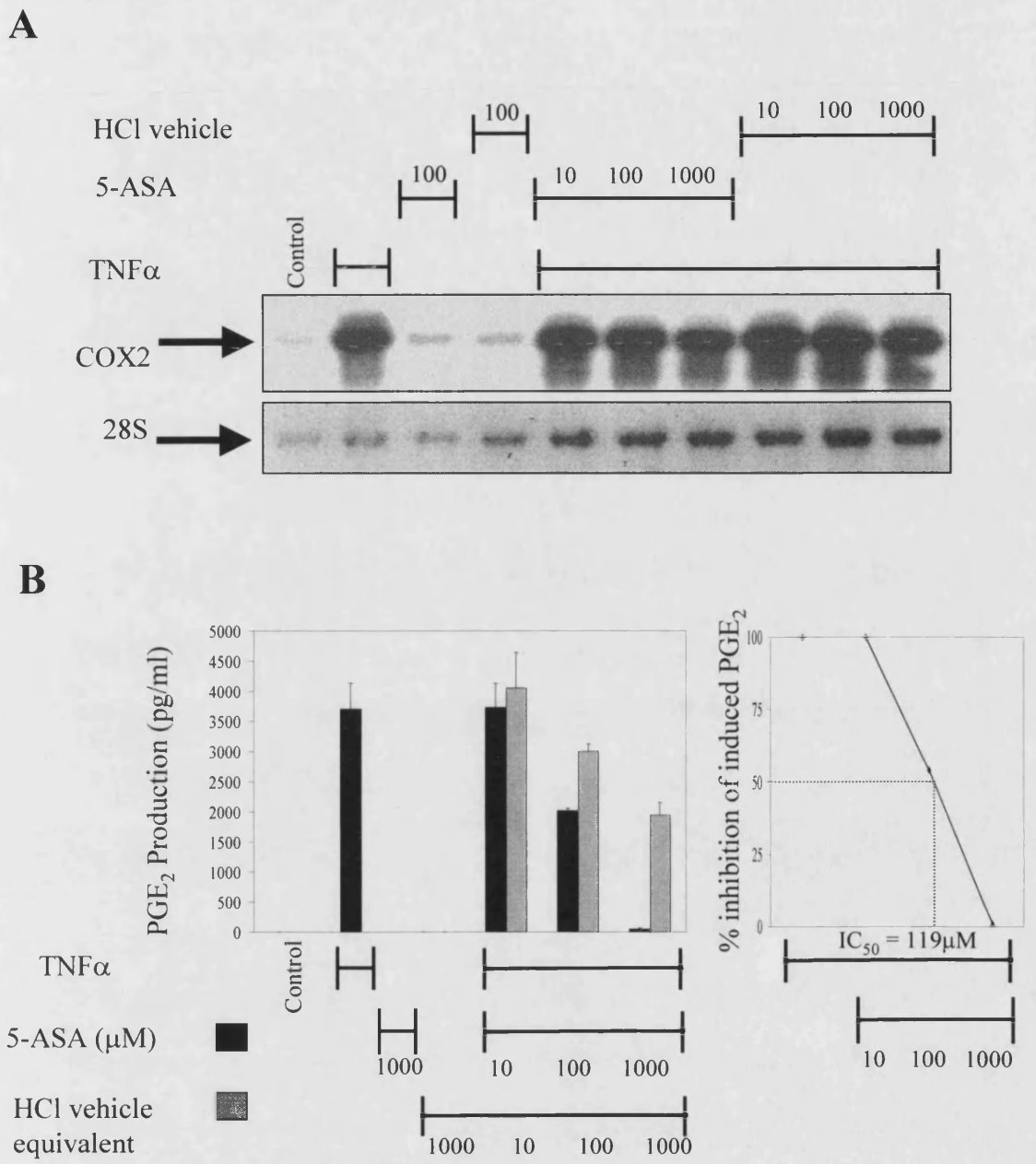


Figure 4.10: ***Regulation of COX2 expression by 5-ASA.***

A. Northern analysis of mRNA isolated from HT-29 cells stimulated with TNFα (100ng/ml) for two hours in the presence or absence of pre-treatment with increasing concentrations of 5-ASA (0-1000μM) or its HCl vehicle equivalent, and probed for COX2. The 28S band is shown to demonstrate loading. **B.** Bar chart representing PGE_2 production by HT-29 cells stimulated in triplicate by TNFα (100ng/ml) for 72 hours in the presence or absence of pre-treatment with increasing concentrations of 5-ASA (0-1000μM) or its HCl vehicle equivalent. Blots were from single experiments but are representative of one other.

4.3 Results Summary

- The agents shown to induce COX-2 are as outlined in the Table below. LPS and IFNγ failed to induce COX-2 in either HT-29 or Caco-2 cells.

	Induced COX-2	**TNFα**	**IL-1**
HT-29	*mRNA*	✓	✓
	Protein	✓	✓
	PGE_2 product	✓	✗
	PGD_2 product	✗	✗
Caco-2	*mRNA*	✗	✓
	Protein	✗	✓
	PGE_2 product		✗
1° IECs	*mRNA*		✗
	Protein		✗

- PGE_2 caused a concentration dependent decrease in TNFα induced COX-2 expression at the mRNA and protein level.
- PPARγ agonists had conflicting effects on TNFα induced COX-2 expression. They had no effect on COX-2 mRNA, caused an up-regulation of COX-2 protein and an inhibition of PGE_2 production.
- 5-ASA caused minimal inhibition of TNFα induced COX-2 mRNA but caused a more marked inhibition of TNFα induced PGE_2 production (IC_{50}=119μM).

4.4 Discussion

The investigation of intestinal epithelial cell biology is limited by the inability to culture and passage freshly isolated primary intestinal epithelial cells (IECs) (Evans *et al.*, 1994). This inability to easily manipulate the cells one wishes to draw conclusions about is despite much work in this area and sporadic reports of limited success in supporting fresh human IECs *in vitro* (Brandsch *et al.*, 1998; Rogler *et al.*, 1998). However, freshly isolated primary human IECs cannot reliably be supported in culture and, after a four hour isolation process which includes mechanical and enzymatic disruption, only remain viable for at most 24 hours. This does allow some simple experiments to be performed as shown here but there is the concern that, although the cell type is fresh and human, the results may be artefactual due to the isolation process.

As a result of this, researchers have come to depend on a number of transformed cell lines which are usually derived from human adenocarcinomas. They are readily available and easily grown and passaged, have a characteristic genotype and phenotype (albeit with marked differences from normal human cells) and their use is widely reported in the relevant scientific literature. Ideally reports of findings in cell lines can then be corroborated by work in primary IECs.

Three cellular models were used to explore COX-2 expression in intestinal epithelial cells; two transformed intestinal epithelial cell lines – HT-29 and Caco-2 – along with freshly isolated primary human intestinal epithelial cells. The HT-29 cell line was established from a colonic adenocarcinoma in a 44 year old caucasian female in

1964 while the Caco-2 cell line was established in 1974 from a similar adenocarcinoma in a 72 year old caucasian male (Rousset, 1986). Although both cell lines are established models of intestinal epithelial cells, the main difference between them is that HT-29 are generally less differentiated than Caco-2 cells.

This work looks at COX-2 expression at the mRNA, protein and product level. To investigate COX-2 mRNA expression northern analysis was used as opposed to RT-PCR as a more direct technique with less assumptions and allowing better comparison between samples. Initial development of a COX-2 cDNA probe for northern analysis was carried out using Digoxigenin labelling, but lack of reproducibility forced the change to radioactive labelling of commercially available COX-2 cDNA with [^{32}P] (Soriani *et al.*, 1999). This gave reproducible and clear results for COX-2 and was the technique adopted throughout the work shown here.

The switch to [^{32}P] labelling did not help improve the quality of COX-1 northern blots as conditions could not be found, using this technique, to allow consistent reproducible COX-1 mRNA analysis. Although COX-1 has occasionally been shown to be inducible in some systems, and inducible in the gastrointestinal tract by irradiation (Cohn *et al.*, 1997), there was no evidence in this work or in the work of others that COX-1 is inducible by cytokines in any intestinal model of epithelial cell function.

Both the colorectal carcinoma derived cell lines used display a small amount of constitutive COX-2 expression at both the mRNA and protein level which is faintly seen in blots with good sensitivity (Figures 4.10 and 4.9 for mRNA and protein

respectively in HT-29 cells; Figure 4.3 for Caco-2 cells). This is in keeping with results from other groups (Tsujii and DuBois, 1996; Tsujii *et al.*, 1997; Battu *et al.*, 1998; Hsi *et al.*, 2000), and that the origin of these transformed cell lines is from colorectal cancers. However the small amount of constitutive expression seen is not reflected in detectable amounts of PGE_2 production.

It has been demonstrated here that the pro-inflammatory cytokines TNFα, IL-1α and IL-1β can induce COX-2 in intestinal epithelial cell lines. This is not especially surprising as these cytokines have been shown to induce COX-2 in a number of systems and have been shown to induce other responses in intestinal epithelial cells (Eckmann *et al.*, 1997; Jobin *et al.*, 1998; Kolios *et al.*, 1999). However there are a few features of these results which are surprising.

Firstly, regarding HT-29 cells, these cells are known to have TNFα and IL-1 receptors and ligand binding has been shown to activate NFκB which is essential to COX-2 transcription (Jobin *et al.*, 1998; Jobin *et al.*, 1999). HT-29 cells have also been shown to have mRNA and protein for the LPS receptor CD14 (Cario *et al.*, 2000) and can respond to LPS activation by activating NFκB as well as inducing IL-8 generation (Toshina *et al.*, 2000; Schuerermaly *et al.*, 1994), a chemokine known to be NFκB dependent in these cells (Jobin *et al.*, 1998). Surprisingly, LPS did not activate COX-2 in this system, a result which is not contradicted in the literature. When considered with published results for COX-2 induction (Jobin *et al.*, 1998), this implies that activation of NFκB is necessary but not sufficient for COX-2 induction in HT-29 cells.

Inducing Agent	Cell type	Reference
TNFα	HT-29 Caco-2	(Eckmann *et al.*, 1997) (Stenson *et al.*, 1993)
Bacterial Invasion	HT-29	(Eckmann *et al.*, 1997)
IGF-II	Caco-2	(Di Popolo *et al.*, 2000)
Dihydroxy Bile Acids	SK-GT-4 HCT 116	(Zhang *et al.*, 1998) (Glinghammar and Rafter, 2001)

Table 4.1: ***Agents proven to induce COX-2 in intestinal epithelial cell lines***

A second point of interest concerning COX-2 induction in HT-29 cells is the observed lack of effect of IFNγ (Results Figure 4.2). IFNγ has been shown to induce COX-2 in other systems (Matsuura *et al.*, 1999). It has also been shown to be necessary in the induction of iNOS in HT-29 cells, an enzyme that shares many features with COX-2, although in that context it has to work in synergy with IL-1α (Kolios *et al.*, 1995). Although regarded as part of the Th1 response, IFNγ is an immunomodulatory cytokine, as opposed to a classic pro-inflammatory cytokine such as TNFα, and its regulatory properties on TNFα induction of COX-2 are further explored in chapter 5.

The COX-2 dependent induction of PGE_2 by TNFα in HT-29 is a logical sequelae of its marked induction at the level of mRNA and protein. However this is at odds with Hsi *et al* who report that the COX-2 found in HT-29 cells is catalytically inactive

and unable to result in PGE_2 synthesis despite no mutation in the COX-2 gene (Hsi *et al.*, 2000). This is further confounded by the fact that they cloned HT-29 derived COX-2 into HCT-116 IECs and the cloned COX-2 was active. Such results are confusing and an explanation may be in the fact that these workers did not use cytokine stimulation, and perhaps the TNFα is having an additional post-translational effect. The work presented here, which is supported by the literature (Jobin *et al.*, 1998), would seem to prove the HT-29 COX-2 can synthesise PGE_2 when in the presence of TNFα. Indeed, it may be that there needs to be a critical amount of COX-2 protein to allow PGE_2 production. IL-1α caused a less marked induction of COX-2 mRNA and protein with no resulting PGE_2 synthesis. Similarly, no PGE_2 production was seen in Caco-2 cells where the equivalent induction of COX-2 was smaller. Against this is the fact that Hsi *et al* report PGE_2 synthesis by unstimulated Caco-2 cells, something that was not seen during this work (Hsi *et al.*, 2000).

Finally, concerning HT-29 cells and COX-2 induction, is the lack of induction of PGD_2 production as assessed by measuring its stable methoximated derivative by ELISA. As a contrasting prostaglandin to PGE_2, it is not known whether the intestinal epithelium can synthesis PGD_2, or whether the conditions for its synthesis have not been met, and there is no supporting evidence in the literature.

With respect to Caco-2 cells, the response to IL-1α is again not unexpected. However the lack of clear response to TNFα is surprising as it is known that these cells can respond in a number of ways to TNFα including generation of IL-8 (Toshina *et al.*, 2000), IL-1β (Chowers *et al.*, 2001) and MMP-9 (Gan *et al.*, 2001).

One would expect the IL-8 response to be NFκB dependent although activation of NFκB in Caco-2 cells by TNFα has not been demonstrated. Secondly, as with HT-29 cells, no induction of COX-2 was seen in response to LPS in Caco-2 cells. However, there is evidence that these cells may not be able to respond to LPS (Toshina *et al.*, 2000). In contrast, the lack of observed response to IFNγ in Caco-2 cells may mirror the situation in HT-29 cells as Caco-2 cells do have the ability to respond to IFNγ (Cavicchi and Whittle, 1999).

Having established the characteristics of COX-2 induction in two *in vitro* cell lines supportive evidence was sought in primary intestinal epithelial cells (IECs). As previously mentioned, intestinal epithelial research is limited by the difficulties in isolating and then subsequently culturing primary cells. IECs have been shown to have receptors for IL-1, and also respond to IL-1β (Jobin *et al.*, 1998), and this was therefore chosen as an inducing agent. However, there was no evidence of induction of COX-2 mRNA or protein with this cytokine. One possible explanation concerns the cell population stimulated. The technique used to isolate IECs was one widely used in the literature. However, the final cell population was not assessed by FACS (Fluorescent Activated Cell Sorting) analysis to confirm a uniform epithelial cell population. A second option is that this is a true result and that there are significant differences between IECs and the *in vitro* models used to investigate their biology. This would be supported by the absence of TNFα receptors on IECs compared to their presence on many intestinal epithelial cell lines (Panja *et al.*, 1998). A third possible explanation is that this isolation process, which results in cell death by 24 hours, causes changes in cell physiology rendering them either unresponsive, or with markedly altered responses from those of their normal physiology. On the basis of

the accepted difficulties of working with IECs in this area of research, it was felt that drawing reliable conclusions from such work was not possible and the use of IECs was not pursued.

The regulation of COX-2 by downstream products, such as PPARγ agonists (PGJ_2) and PGE_2, raises many issues particularly concerning PGE_2 which is a known product of COX-2 in this system. PGE_2 is shown to negatively regulate induced COX-2 mRNA and protein at concentrations chosen because they reflect the amount of PGE_2 induced by cytokine stimulation. They also represent concentrations which are similar to those reported in patients with UC (Sharon *et al.*, 1978). This supports the fact that these results are likely to be physiologically relevant with the induced PGE_2 production providing negative feedback for COX-2. This would effectively help limit the COX-2 dependent response. Evidence for a possible mechanism of action for this effect comes from recent work on the signalling pathways activated by PGE_2 (Sheng *et al.*, 2001). The binding of PGE_2 to its EP4 receptor has been shown to activate the PI 3-Kinase signalling cascade and its downstream effector PKB. Evidence presented in chapter 6 points to a negative regulatory role for PI 3-Kinase on COX-2 expression (Weaver *et al.*, 2001), thus providing a possible mechanistic pathway of action (Figure 4A). However this has not been rigorously proven and there is conflicting evidence from PGE_2 binding causing an increase in cAMP and a resulting positive effect on COX-2 expression (Fournier *et al.*, 1997; Inoue *et al.*, 2000). Such a hypothesis does not fit with the results presented here but may be playing a contributory role albeit with the net effect one of COX-2 inhibition.

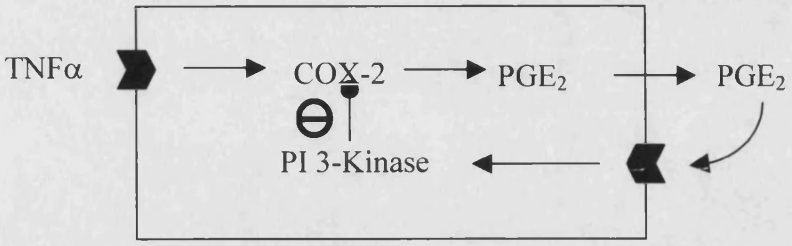


Figure 4A: ***Schematic representation of possible mechanism for PGE_2 negatively regulating COX-2 via a PI 3-Kinase dependent pathway.***

Although the synthesis of PGD_2, a precursor of cyclopentenone prostaglandins, could not be demonstrated in this system, there is evidence that such potential COX-2 products can regulate COX-2 expression and activity. The use of two differing PPARγ agonists reveals consistent results with contrasting interpretations. Both ciglitazone, a thiazolidinedione class drug, and PGJ_2, reported as being a physiological agonist, both increase COX-2 protein when used alone or in conjunction with TNFα stimulation. However they both inhibit TNFα induced PGE_2 production with PGJ_2 being more effective than ciglitazone. Both aspects of these results receive support from the literature and it is obviously possible to have conflicting regulation at different points of the RNA to protein to product pathway, a theme which is explored in chapter 5. In this context it is probably most valid to consider the functional readout of COX-2, that is its enzymatic activity to synthesise PGE_2, as the best marker of PPARγ agonist stimulation. Inhibition of PGE_2 production is supported by work in a macrophage cell line (Inoue *et al.*, 2000). Also, with the hypothesis that COX-2 expression and PGE_2 production in this acute model is generally pro-inflammatory, would be supported by the generally reported anti-inflammatory actions of PPARγ on chemokine generation, iNOS activation and macrophage activation (Jiang *et al.*, 1998; Ricote *et al.*, 1998; Momoi *et al.*, 1999

Petrova *et al.*, 1999). It is believed that PPARγ agonists exert their effects at least in part by inhibiting the NFκB pathway by direct inhibition of IκB Kinase (Rossi *et al.*, 2000). This would correlate with a general anti-inflammatory profile and inhibiton of COX-2. However, in this context one would expect to see inhibition of COX-2 transcription with decreased mRNA levels. Also, NFκB inhibition is at odds with the increase in COX-2 protein and the post-translational effect of decreased PGE_2 production. Therefore there must be additional mechanisms for PPARγ activation to mediate its effects in this system.

Finally it should be pointed out that regulation of COX-2 by its products may involve altering the prostaglandin profile produced rather than altering COX-2 mRNA or protein levels *per se*. This system lacks the sophistication to assess this, due to lack of a second prostaglandin as a functional readout of COX-2 activity. Regulation of differing prostaglandin synthases may explain some of the contrasting evidence as is particularly seen with the PPARγ agonist results.

The regulation of prostaglandin production by 5-ASA in models of IBD is an important clinical question as this is a common therapeutic agent in IBD in general and colitis in particular. 5-ASA is related to aminosalicylates, but is not a non-steroidal anti-inflammatory drug (NSAID), and has been documented as having many potentially beneficial actions in IBD (Greenfield *et al.*, 1993). It has been shown to inhibit prostaglandin production when used in the mM concentration range (Sharon *et al.*, 1978; Hawkey and Truelove, 1983; Greenfield *et al.*, 1993;). In contrast, it promotes prostaglandin production in the μM concentration range (Hawkey *et al.*, 1985; Greenfield *et al.*, 1993;), which raises the important question

of which concentration of 5-ASA is relevant to its use in the treatment of IBD (De Vos *et al.*; 1992Riley, 1998). The measurement of 5-ASA in the colon is challenging and there is debate about which site is the most appropriate to measure; the lumen, mucosa or serum? Taking a consensus view that the μM range was most physiologically appropriate, 5-ASA was used to assess regulation of COX-2, rather than the previous work looking solely at prostaglandin synthesis often done prior to the discovery of inducible cyclo-oxygenase. This reveals that 5-ASA only inhibits COX-2 mRNA once in the mM range, and even then only slightly, and that the IC_{50} for inhibition of PGE_2 production was 119μM. At 10μM 5-ASA there was no apparent regulatory action on COX-2 mRNA or PGE_2 production.

The optimal therapeutic concentration for 5-ASA is crucial to interpreting these results. If it is in the low μM range then 5-ASA is unlikely to be exerting any effects via COX-2 inhibition. However, if it is higher then this may be a relevant action of 5-ASA in IBD although it may not be beneficial to chronic intestinal inflammation as inhibition of COX exacerbates IBD, and inhibition of COX-2 exacerbates animal models of this disease (Reuter *et al.*, 1996). Furthermore, there is evidence that 5-ASA may decrease the risk of malignant transformation (Brown *et al.*, 2000). Such an action could be related to chronic COX-2 inhibition as is seen with decreased rates of colorectal carcinoma in long term users of NSAIDs (Giovannucci *et al.*, 1994; Giovannucci *et al.*, 1995). In summary, because of the many actions of 5-ASA, and the uncertainties about the relevant therapeutic concentration, theories concerning its detailed action can only be speculative.

5 Results – Regulation of Induced COX-2 by Cytokines

5.1 Introduction

Having established that the pro-inflammatory cytokines TNFα and IL-1 α or β can induce COX-2 mRNA in intestinal epithelial cells, the next aim was to assess any possible regulatory role for other cytokines relevant to gastrointestinal biology and disease. In particular, although Interferon-γ (IFNγ) had no effect on COX-2 induction in isolation (chapter 4), the possibility of an interaction with TNFα or IL-1α was addressed. IFNγ has been shown to be raised in IBD and has been shown to induce COX-2 in human keratinocytes (Matsuura *et al.*, 1999). Furthermore it has been demonstrated to interact synergistically with TNFα to induce chemokines in HT-29 intestinal epithelial cells (Warhurst *et al.*, 1998). In contrast, it has been shown to inhibit IL-1β induced COX-2 transcription in human macrophages (Barrios-Rodiles and Chadee, 1998).

The second aim was to explore the possible immunoregulatory role of Th2 cytokines such as IL-4, IL-10 and IL-13. Although HT-29 cells appear to lack the machinery to respond to IL-10 (Bourreille *et al.*, 1999), IL-4 and IL-13 have been shown to inhibit pro-inflammatory cytokine activation of iNOS, as well as chemokine production, in HT-29 cells (Wright *et al.*, 1997; Kolios *et al.*, 1999). With respect to the regulation of COX-2, there are reports of an inhibitory role of Th2 cytokines in other non-gastrointestinal systems.

5.2 Results

5.2.1 Regulation of cytokine induced COX-2 by Interferon-γ

5.2.1.1 Interferon-γ and COX-2 mRNA

To investigate the effect of IFNγ on the cytokine induction of COX-2 mRNA, HT-29 cells were simultaneously stimulated with TNFα or IL-1α in the presence of increasing concentrations of IFNγ (0-300u/ml) for two hours before isolation of mRNA for northern blot analysis and probing for COX-2 (Figure 5.1A and 5.1B). IFNγ inhibited the induction of both TNFα and IL-1α induced COX-2 mRNA, and this inhibition was concentration dependent.

Having established an inhibitory action of IFNγ on induced COX-2 mRNA generation, a possible mechanism of action of IFNγ was explored using the selective JAK2 inhibitor AG490. IFNγ is believed to signal through a receptor complex requiring the activation of JAK2. Once activated it can activate the other receptor associated JAK, JAK1, and then together they phosphorylate the tyrosine containing $_{440}$YDKPH$_{444}$ sequence near the C-terminus of the IFNGR1. This allows the formation of STAT1 docking sites, their subsequent activation and downstream effects (Stark *et al.*, 1998). It would therefore be expected that JAK2 inhibition would inhibit any effects of IFNγ mediated through its receptor. HT-29 cells were stimulated in the presence or absence of simultaneously added IFNγ, with or without pre-treatment with the JAK2 inhibitor AG490 at 100μM for 15 minutes (Figure

5.1C). TNFα was seen to induce COX-2 mRNA which was partially inhibited by 300u/ml IFNγ. Interestingly, pre-treatment with AG490 had no effect on this inhibitory action of IFNγ as well as having no effect when used in isolation (Figure 5.1C).

The next question to address was whether the action of IFNγ on inhibiting total quantities of induced COX-2 mRNA was due to reducing the mRNA stability. COX-2 is known to be regulated in this way via the adenylate and uridylate rich elements or AREs in the 3′ untranslated region. Indeed, it has been shown that the majority of the increase seen in COX-2 mRNA post induction is due to increased stability rather than increased transcription (Huang *et al.*, 2000). To assay COX-2 mRNA stability, Actinomycin D chase experiments were performed. Actinomycin D (AcD) prevents RNA synthesis and this is demonstrated in Figure 5.2A. HT-29 cells were pre-treated for one hour with increasing concentrations of AcD (5-20μg/ml) and then stimulated with TNFα at 100ng/ml. When compared to a positive control without AcD pre-treatment, it can be seen that AcD prevents TNFα induced COX-2 mRNA generation at all concentrations (Figure 5.2A). To perform a chase experiment, HT-29 cells were stimulated with a cytokine for two hours (initially TNFα at 100ng/ml), and then AcD was added preventing any new mRNA synthesis. The mRNA synthesised in the first two hours then decays at a rate depending on its stability. Figure 5.2B demonstrates that this occurs similarly for AcD at concentrations of 5-20μg/ml and therefore 5μg/ml was subsequently used throughout. AcD on its own had no effect on COX-2 induction (Figure 5.2D) and the vehicle for AcD, DMSO, did not alter the cytokine induction of COX-2 mRNA in any way (Figures 5.2C and D).

HT-29 cells, in the presence or absence of IFNγ, were stimulated for two hours with TNFα. At two hours AcD was added to give a concentration of 5μg/ml and mRNA was then isolated at this point and at one to six hours thereafter and analysed for COX-2 mRNA by Northern blot analysis. The decay of signal seen on Northern blot analysis allows the calculation of a half life for COX-2 mRNA. For this series of experiments the half life of TNFα induced COX-2 mRNA was shown to be about 3 hours and this was not significantly altered by the presence of IFNγ (Figure 5.3)

5.2.1.2 Interferon-γ and TNFα induced COX-2 protein and PGE_2 product

Having established an inhibitory effect of IFNγ on TNFα or IL-1α induced COX-2 mRNA in HT-29 cells, the work progressed to assess the effects of IFNγ on TNFα induced COX-2 protein. HT-29 cells were stimulated for six hours with increasing concentrations of TNFα alone (30 and 100ng/ml) and in combination with IFNγ (300u/ml). Subsequent western blot analysis for COX-2 shows that the induction of COX-2 protein by TNFα is concentration dependent (Figure 5.4A). The additional presence of IFNγ caused a further significant increase in COX-2 protein compared to TNFα alone. This synergistic action between TNFα and IFNγ on COX-2 protein induction is surprising considering the opposite action of IFNγ at the level of COX-2 mRNA.

It was then explored, as with the work on IFNγ inhibition of induced COX-2 mRNA, whether the mechanism of action of IFNγ on further inducing COX-2 protein was mediated via a JAK2 dependent pathway. HT-29 cells were stimulated with TNFα

and IFNγ for six hours in the presence or absence of increasing concentrations of the specific JAK2 inhibitor AG490 (10 and 100μM). Subsequent western analysis of isolated protein showed that the marked induction of COX-2 protein by TNFα and IFNγ was partially inhibited by AG490 at the higher concentration of 100μM (Figure 5.4B). Because AG490 is dissolved in DMSO, a reciprocal experiment using this DMSO vehicle at appropriate dilutions to represent differing concentrations of AG490, was carried out and the membrane probed for COX-2 protein. This showed no effect of DMSO vehicle on TNFα and IFNγ induced COX-2 protein (Figure 5.4C).

Finally, regarding COX-2 protein, it was important to ensure that the AG490 was specifically inhibiting only the super-induction effect of IFNγ and not the basic induction by TNFα alone. HT-29 cells were stimulated with TNFα and IFNγ both alone and in combination, along with TNFα in combination with AG490 pre-treatment (100μM for one hour), and protein analysed for COX-2 by western blot analysis (Figure 5.4D). This demonstrated that AG490 did not effect the induction of COX-2 by TNFα alone and confirmed that IFNγ alone had no inductive effect on COX-2 protein.

Considering the conflicting regulatory action of IFNγ on induced COX-2 at the mRNA and protein level, the regulatory action of IFNγ on the PGE_2 generation, as a functional activity of COX-2, was assessed. HT-29 cells were stimulated with TNFα and increasing concentrations of IFNγ for 24 hours and supernatants collected for measurement of PGE_2 (Figure 5.5). Increasing concentrations of IFNγ caused a

significant, concentration dependent, synergistic increase to TNFα induced PGE_2 production. This super-induction of PGE_2 production by the combination of TNFα and IFNγ was COX-2 dependent as demonstrated by the total inhibition by NS-398, a specific COX-2 inhibitor.

Finally it was investigated whether JAK2 inhibition abrogated the synergistic action of IFNγ with TNFα at this level of COX-2 functional activity as well as at COX-2 protein production. HT-29 cells were stimulated with TNFα and IFNγ for 24 hours in the presence or absence of AG490 and the supernatants were assayed for PGE_2. JAK2 inhibition with AG490 significantly decreased PGE_2 production in a concentration dependent manner with 100μM causing 70% inhibition ($p<0.01$)(Figure 5.6). This inhibitory action was compounded by an effect of the DMSO vehicle not seen at the level of protein and mRNA. However, the inhibitory action of AG490 is greater than that of its vehicle at comparable amounts, and this difference is significant at 100μM ($p<0.05$).

5.2.2 Regulation by Th2 Cytokines

5.2.2.1 Th2 cytokines and HT-29 cells

In view of the proven role of Th2 cytokines in regulating iNOS and C-C chemokines in HT-29 cells (Wright *et al.*, 1997; Kolios *et al.*, 1999), as well their regulation of COX-2 in other non-gastrointestinal systems, there was good evidence to suggest a regulatory role on COX-2 in HT-29 cells. HT-29 cells pre-treated with IL-4, IL-10 or IL-13 (all at 30ng/ml for 1 hour), prior to stimulation with TNFα for two hours,

showed that IL-4 and IL-13, but not IL-10, caused significant inhibition of TNFα induced COX-2 mRNA (Figure 5.7A).

In view of the demonstration that IL-4 and IL-13 have a marked inhibitory action on TNFα induced COX-2 mRNA it was evaluated whether similar effects were observed at the level of protein expression. Indeed, western blot analysis, using a specific anti-COX-2 antibody, revealed that IL-4 and IL-13 again caused a marked inhibition of TNFα induced COX-2 protein, with IL-10 having no effect (Figure 5.7B).

Finally, the action these cytokines had on COX-2 enzyme functional activity, as measured by PGE_2 production was assessed. Again IL-4 and IL-13 were shown to significantly inhibit TNFα induced PGE_2 production ($p<0.01$). The effect of IL-10 was not significant (Figure 5.7C).

5.2.2.2 Th2 cytokines and Caco-2 cells

To explore whether the actions of Th2 cytokines in inhibiting COX-2 induction in HT-29 cells were representative of other intestinal epithelial cells, their action on induced COX-2 mRNA was investigated in Caco-2 cells. Using a similar protocol to that used in HT-29 cells, Caco-2 cells were pre-treated with IL-4, IL-10 or IL-13 (all at 30ng/ml for 1 hour), prior to stimulation with IL-1α for two hours. mRNA was then isolated and probed for COX-2 using northern blot analysis. In contrast to work with HT-29 cells, this showed no alteration of IL-1α induced COX-2 mRNA by IL-4, IL-10 or IL-13 (Figure 5.8).

5.2.2.3 IL-4 and IL-13 activate PKB in HT-29 cells

Finally, with regard to cytokine regulation of COX-2, previous work carried out at the University of Bath has shown that IL-13 activates the signalling pathway PI 3-Kinase and that is how it mediates its inhibition of iNOS (Wright *et al.*, 1997). Although it is known that IL-4 and IL-13 share similar receptor mediated signalling pathways, and that they can both activate PI 3-Kinase in other systems, it has not been shown that IL-4 and IL-13 have the same action in the HT-29 cell line. In particular, it has not been shown that IL-4, like IL-13, can activate PI 3-Kinase dependent signalling cascades. Hence, the effect of IL-4, IL-10 and IL-13 on the activation by phosphorylation of PKB, a downstream effector of PI 3-Kinase which is commonly used as a marker of PI 3-Kinase activation, was assessed. This was done by western blot analysis using a phospho-specific antibody against the phospho-Ser^{473} within the PKB activation loop. HT-29 cells were treated in the presence of the three Th2 cytokines, IL-4, IL-10 or IL-13, over a short time course (0-30 minutes). Cells were lysed, immunoprecipitated with an antibody targeted against PKB using an epitope common to both phosphorylated (active) and unphosphorylated (inactive) forms of the protein, and associated proteins were separated by SDS-PAGE. After transfer to nitrocellulose the separated proteins were probed with an anti-phospho-Ser^{473} PKB antibody, with the same blot being stripped and reprobed for pan PKB to demonstrate equal loading (Figure 5.9). There was some constitutive PKB activation, which was to be expected in this cell line which has been shown to have constitutive PI 3-Kinase activity and is derived from a human colorectal carcinoma (Wright *et al.*, 1997). However, above and beyond this, there was activation of PKB by both IL-4 and IL-13, supporting the prior assumption

that both cytokines share the activation of PI 3-Kinase as a signalling pathway common to both.

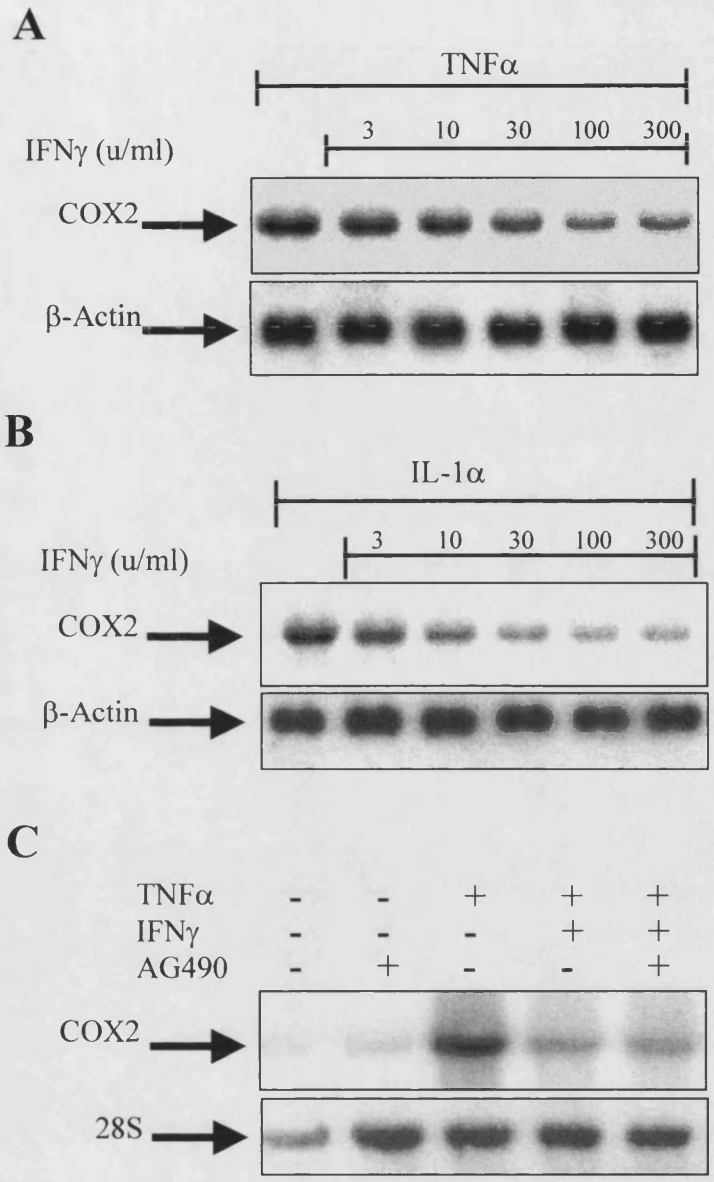


Figure 5.1: ***Inhibition of cytokine induced COX2 mRNA by Interferon-γ.***
A and B. Northern analyses of mRNA isolated from HT-29 cells, which have been stimulated with TNFα (100ng/ml) (**A**) or IL-1α (10ng/ml) (**B**) for two hours having been pre-treated with increasing concentrations of IFNγ (0-300u/ml) and probed for COX2 (upper panels). The membranes were stripped and reprobed for β-Actin to demonstrate loading (lower panels). **C**. Northern analysis of mRNA from HT-29 cells, which have been stimulated as described for two hours (TNFα at 100ng/ml; IFNγ at 300u/ml; AG490 at 100μM one hour prior) and probed for COX2 (upper panel). The 28S band from the membrane prior to probing is shown to demonstrate equal loading. Blots were from single experiments but are representative of at least three others.

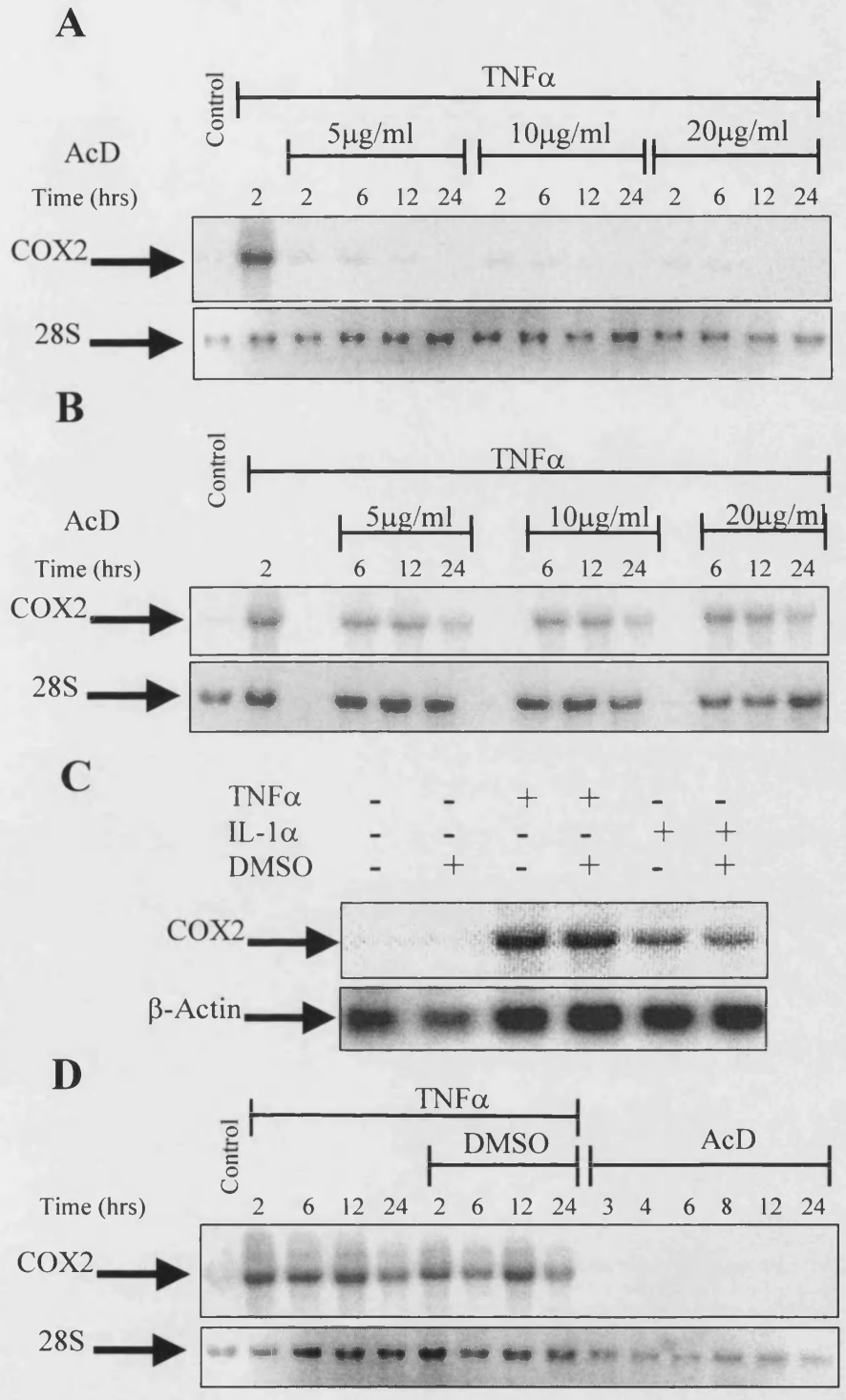


Figure 5.2: ***Inhibition of mRNA synthesis by ActinomycinD, and DMSO control blots***
A to D. Four Northern analyses of mRNA isolated from HT-29 cells and probed for COX2 (upper panels). **A and B**. Cells were stimulated with TNFα (100ng/ml) over a 24 hour time course, having been either pre-treated for one hour with increasing concentrations of Actinomycin D (AcD) (5-20μg/ml) (**A**), or with AcD added two hours after TNFα (**B**). The 28S band demonstrates equal loading. **C**. Cells were stimulated for two hours with TNFα (100ng/ml) or IL-1α (30ng/ml) in the presence or absence of the vehicle for both AcD and AG490, DMSO (10μl (0.005%v/v) - equivalent to 5μg/ml AcD or 200μM AG490). The membrane was stripped and reprobed for β-Actin to demonstrate loading. **D**. mRNA was isolated from cells over a 24 hour time course, having been stimulated by TNFα (100ng/ml) in the presence or absence of DMSO (10μl), or treatment with AcD alone. The 28S band demonstrates loading.

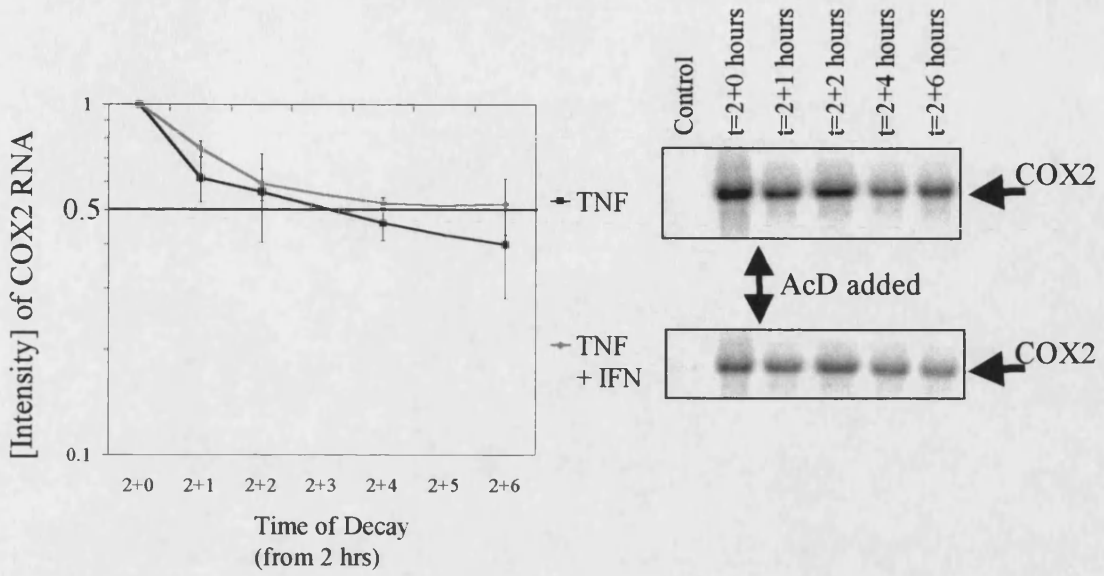


Figure 5.3: ***Decay of COX2 mRNA in the presence or absence of interferonγ***
Logarithmic line plot of [Intensity] of COX2 mRNA as measured by Northern analyses (arbitrary units where maximum intensity is 1) vs time in hours. HT-29 cells were stimulated with TNFα (**A**) for 2 hours in the presence or absence of IFNγ (300u/ml). At 2 hours Actinomycin D (5μg/ml) was added to prevent new RNA synthesis. The subsequent decay in the mRNA signal was plotted to allow calculation of the half life - time at which Intensity is 0.5. The blots shown are representative of three experiments. The membranes were photographed to show the 18S and 28S bands to ensure equal loading (not shown for clarity).

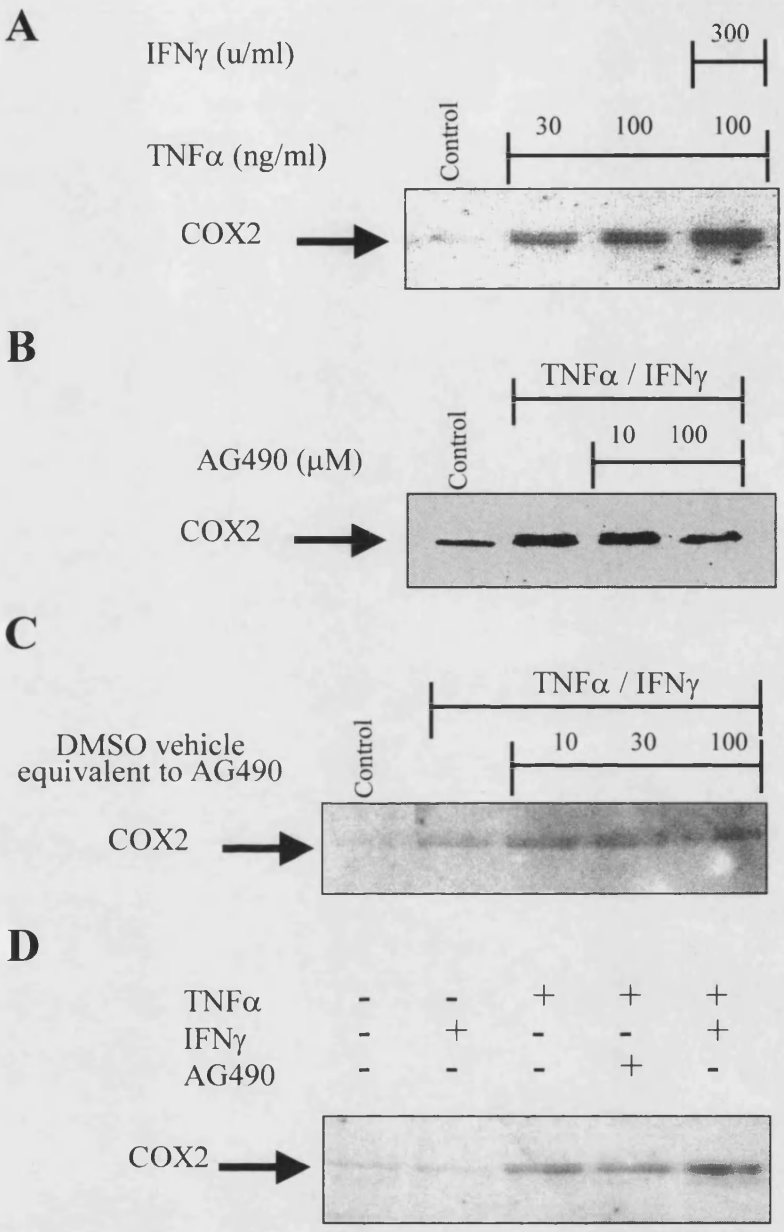


Figure 5.4: ***Synergy between TNFα and IFNγ***

A to D. Western analyses of protein isolated from HT-29 cells and probed for COX2. **A**. Cells were treated for six hours with increasing concentrations of TNFα (0-100ng/ml) and then in combination with IFNγ (300u/ml) added simultaneously. **B**. Cells were treated for six hours with combined TNFα (100ng/ml) and IFNγ (300u/ml) in the presence or absence of pre-treatment with the JAK2 inhibitor AG490 (at 10 and 100μM for one hour). **C**. Cells were treated for six hours in the presence or absence of the AG490 vehicle, DMSO, at concentrations equivalent to AG490 (10-100μM - 0.00025% to 0.0025% v/v). **D**. Cells were treated with TNFα (100ng/ml) and IFNγ (300u/ml), either alone or in combination for six hours. TNFα treated cells also had pre-treatment with AG490 (100μM for one hour).

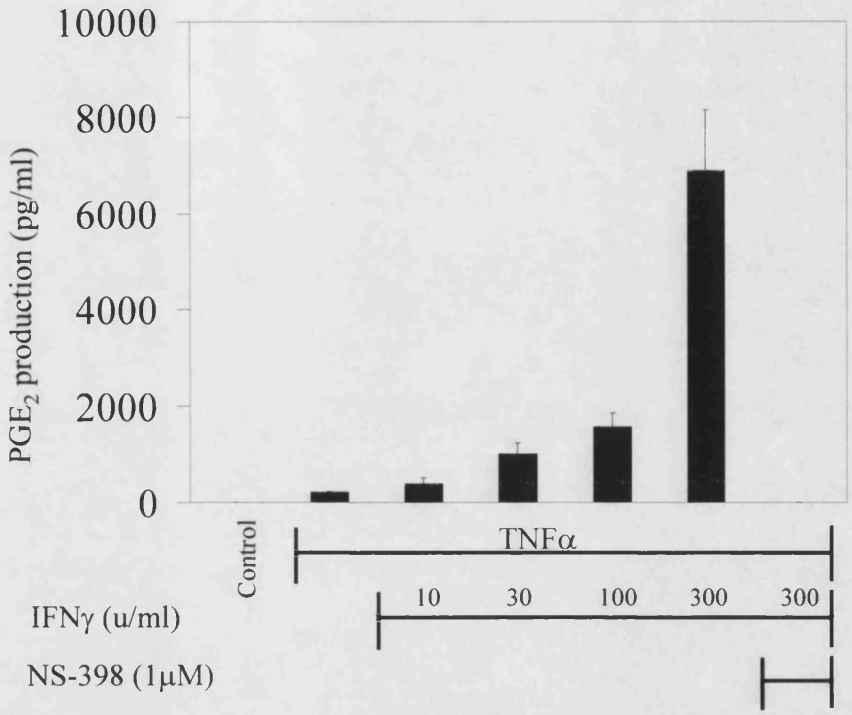


Figure 5.5: ***Synergy between TNFα and IFNγ***

Bar chart of PGE_2 production (pg/ml) stimulated by TNFα (100ng/ml) for 24 hours in the presence of increasing concentrations of IFNγ (0-300u/ml). The combination of TNFα and IFNγ (300u/ml) was pre-treated with the specific COX2 inhibitor, NS-398 at 1μM for one hour to demonstrate COX2 dependence of induced PGE_2 production.

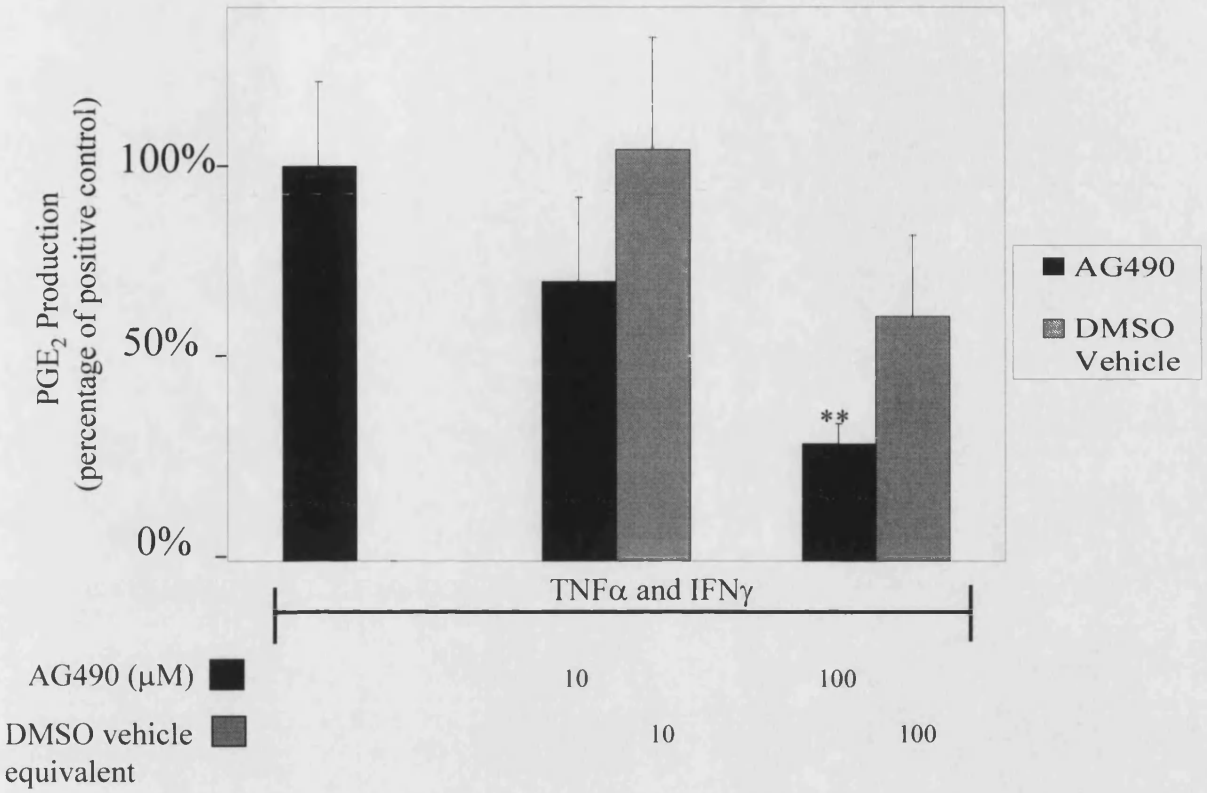


Figure 5.6: ***JAK2 inhibition of IFNγ super-induction of TNFα induced PGE_2***
Bar chart of PGE_2 production (pg/ml) stimulated for 24 hours with TNFα (100ng/ml) and IFNγ (0-300u/ml) in the presence of increasing concentrations of the specific JAK2 inhibitor AG490 (10-100μM) or equivalent concentrations of its DMSO vehicle. ** represents a significant inhibition of PGE_2 production ($p<0.01$).

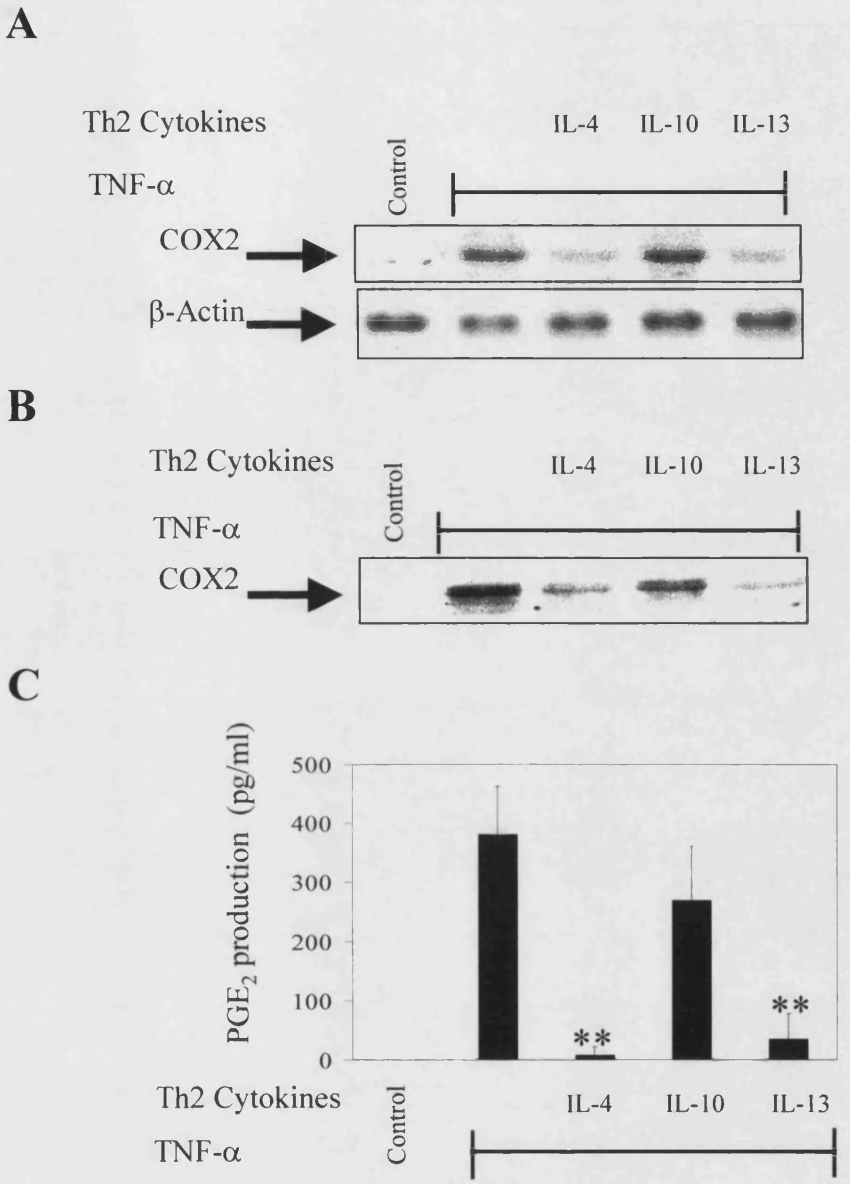


Figure 5.7: ***Regulation of TNFα induced COX2 expression by Th2 cytokines.***
A. Northern analyses of mRNA isolated from HT-29 cells, which have been pre-treated with the Th2 cytokines IL-4, IL-10 or IL-13 (30ng/ml for one hour) and stimulated with TNFα (100ng/ml) for two hours (**A**) and probed for COX2 (upper panels). The membrane was stripped and reprobed for β-Actin to demonstrate loading (lower panels). **B**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with TNFα (100ng/ml) having been pre-treated with Th2 cytokines as labelled (30ng/ml for one hour), and probed with a specific anti-COX2 antibody (upper panel). **C**. An ELISA for PGE_2 (pg/ml) using supernatants from HT-29 cells stimulated with TNFα (100ng/ml) for 24 hours having been pre-treated with Th2 cytokines as labelled (30ng/ml for one hour). This is the result of one experiment using triplicate samples and is representative of three other experiments. Blots are from single experiments but are representative of at least three others. ** represents a significant inhibition of PGE_2 production ($p<0.01$).

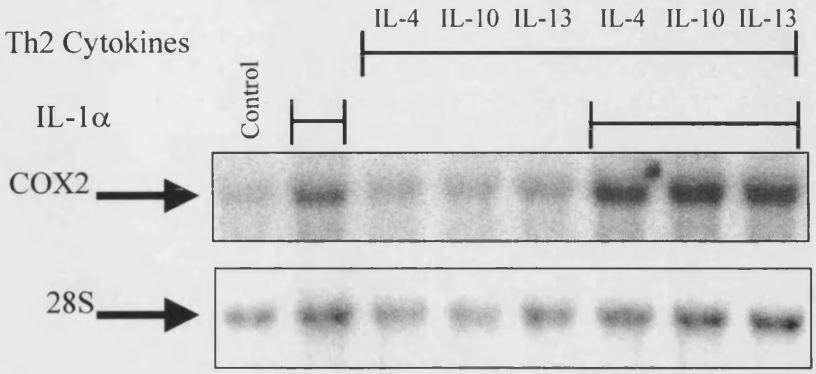


Figure 5.8: ***Lack of regulation of COX2 by Th2 cytokines in Caco-2 cells.***
A. Northern analyses of mRNA isolated from Caco-2 cells, which have been pre-treated with the Th2 cytokines IL-4, IL-10 or IL-13 (30ng/ml for one hour) and stimulated with IL-1α (10ng/ml) for two hours (**A**) and probed for COX2 (upper panels). The 28S band is shown to demonstrate loading (lower panels). Blots are from single experiments but are representative of one other.

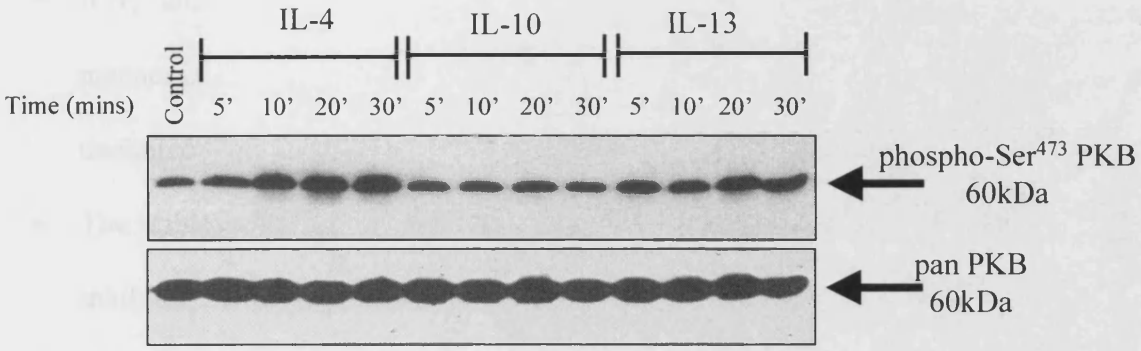


Figure 5.9: ***Activation of PKB by Th2 cytokines.***

A. Western analyses of protein isolated from HT-29 cells, stimulated over a short time course (0-30 mins) with the Th2 cytokines IL-4, IL-10 or IL-13 (30ng/ml). Lysates were immunoprecipitated with specific goat anti-PKB antibody and then probed with a specific anti-phospho-Ser^{473} PKB antibody (upper panels). Membranes were stripped and reprobed for rabbit anti-PKB to demonstrate equal loading. Blots are from single experiments but are representative of one other.

5.3 Results Summary

- IFNγ inhibits the induction of COX-2 mRNA in a concentration dependent manner in HT-29 cells. This inhibition of COX-2 mRNA induction by IFNγ was unaltered by incubation with a JAK2 inhibitor.
- The stability of induced COX-2 mRNA was unaltered by IFNγ implying that its inhibitory action occurs at the level of decreased transcription.
- IFNγ potentiated the induction of COX-2 protein and PGE_2 product in a concentration dependent manner in HT-29 cells. This potentiation was reversed by incubation with a JAK2 inhibitor in a concentration dependent manner.
- The Th2 cytokines IL-4 and IL-13 inhibited the induction of COX-2 mRNA, protein and PGE_2 product in HT-29 cells, whereas IL-10 had no effect.
- In Caco-2 cells IL-4, IL-10 and IL-13 had no effect on COX-2 mRNA induction.
- In HT-29 cells IL-4 and IL-13 induce the phosphorylation, and presumed activation, of the downstream effector of PI 3-Kinase, PKB.

5.4 Discussion

The work presented here demonstrates the many complexities involved in regulating gene expression. The regulatory action of the Th1 cytokine IFNγ on intestinal COX-2 expression has differing effects at the mRNA level when compared with the end points of protein and product. The overall effect of IFNγ when considering functional activity of COX-2 is a synergistic action with TNFα as has been previously described in this system (Arias-Negrete *et al.*, 1995; Warhurst *et al.*, 1998). However the concentration dependent inhibition of COX-2 mRNA gives an insight into the many points of regulating COX-2 expression. There is now evidence of COX-2 being regulated at the transcriptional level to alter RNA synthesis (Reddy *et al.*, 2000), post-transcriptional alteration of RNA stability (Dixon *et al.*, 2000;Huang *et al.*, 2000), changing the translational rate (Dixon *et al.*, 2000) as well as the demonstration of variation between different intestinal cell types of COX-2 protein stability (Shao *et al.*, 2000). Finally the activity of the protein can be altered as is done by specific COX-2 inhibitors.

Looking initially at the level of COX-2 mRNA induction, it can been seen that IFNγ causes a concentration dependent decrease in both TNFα and IL-1α induced COX-2 mRNA. This does not appear to be JAK2 dependent and this is surprising as current theories regarding IFNγ signalling always involve JAK2 (Bach *et al.*, 1997; Stark *et al.*, 1998). The reason for this does not appear to be due to the ineffectiveness of the AG490 compound used as a JAK2 inhibitor as the synergistic activity of IFNγ on TNFα induced COX-2 protein and PGE_2 production was sensitive to this compound.

Alternatively, there may a second confounding action of AG490. Although it is generally regarded as a specific JAK2 inhibitor there is evidence that it may inhibit JAK3, STAT and MAP Kinase dependent pathways in some systems (Wang *et al.*, 1999). However, the possibility of a novel signalling pathway for IFNγ signalling can not be ruled out and there is evolving evidence that there are naturally occuring variations in these signalling pathways (Dupuis *et al.*, 2001).

The mechanism of action of IFNγ inhibiting COX-2 mRNA induction is also interesting. As has been stated, much of the regulation of COX-2 at the mRNA level is by altering mRNA stability rather than necessarily altering transcription (Dixon *et al.*, 2000; Huang *et al.*, 2000). However, in this instance, the presence of IFNγ in the cell culture medium did not alter the stability of the induced COX-2 mRNA as measured by Actinomycin D chase experiments. Other researchers report that IFNγ can inhibit IL-1β induced COX-2 transcription (Barrios-Rodiles and Chadee, 1998). However, in their system using macrophages, these inhibitory effects were also seen at the level of COX-2 protein and PGE_2 production. During this work, attempts were made to measure COX-2 mRNA transcription rates using nuclear run-on assays but unfortunately the assay did not give interpretable results. Thus, the assertion that IFNγ inhibits TNFα induced COX-2 mRNA transcription in this system cannot be proven.

Likely to be of more functional significance is the finding that IFNγ acts synergistically with TNFα with regards to COX-2 protein and PGE_2 production. This has been previously demonstrated in HT-29 cells with respect to the induction of the chemokines RANTES and MCP-1 (Warhurst *et al.*, 1998). Interestingly, their

work showed that such synergy was dependent on the end point assessed, as IFNγ did not potentiate the TNFα induction of IL-8. Such synergy has been demonstrated in an alternative intestinal epithelial cell line, T84 cells (Fish *et al.*, 1999), although there are models outside of the gastro-intestinal system which show the opposite effect (Minghetti *et al.*, 1996; Hewett, 1999). A potential mechanism for the synergistic action of TNFα and IFNγ in the HT-29 cell line is proposed by Blanch *et al* (Blanch *et al.*, 1999). They demonstrate a role for IRF-1 in the regulation of the polymeric immunoglbulin receptor which is coordinately regulated by TNFα/IL-1β and IFNγ. There is supportive evidence showing that IRF-1 regulates COX-2 expression in murine peritoneal macrophages although, as is often seen, the details do not precisely match in cells of an alternative species and type (Blanco *et al.*, 2000).

Th2 cytokines are known to have an immunoregulatory role and have been used with beneficial effects in therapeutic trials of IBD in humans (Van Deventer *et al.*, 1997; Weaver and Robertson, 2000). IL-13 has been shown to down-regulate RANTES and iNOS in HT-29 intestinal epithelial cells and the mechanism for the latter was demonstrated as being dependent on the activation of PI 3-Kinase by IL-13 (Kolios *et al.*, 1999; Wright *et al.*, 1997). In this work we have demonstrated that IL-4 and IL-13 inhibit COX-2 expression and activity in HT-29 cells. Indeed, Th2 cytokines have been reported to inhibit COX-2 expression in a variety of other systems, including osteoblasts (Onoe *et al.*, 1996) and monocytes (Niiro *et al.*, 1998). That IL-10 was not demonstrated to have an effect is unsurprising in a system which appears to be unable to respond to this cytokine (Bourreille *et al.*, 1999; Kolios *et al.*, 1999). IL-10 can bind IL-10 receptors in HT-29 cells but they lack IL-10 receptor

mRNA implying that the IL-10 receptor may be shortened, lacking the intracellular portion necessary to mediate cell responses (Bourreille *et al.*, 1999). There is one paper in the literature citing an action of IL-10 binding in HT-29 cells but it is isolated and is at odds with the apparent deficiencies of the IL-10 signalling pathway in these cells (Schottelius *et al.*, 1998).

It is interesting that there was no regulation of COX-2 mRNA expression by any of the Th2 cytokines in Caco-2 cells. It is worth noting that a different inducing cytokine was used and this may be relevant in this context. However, there is a precedent for IL-4 and IL-13 causing a physiological response in HT-29 cells but not in Caco-2 cells (Gingras and Simard, 1999). This does not appear to be a uniform finding with others reporting that pre-incubation of Caco-2 cells with IL-4 and IL-13 inhibits the cytokine induction of IL-8, an NFκB dependent gene like COX-2 (Lugering *et al.*, 1999). Furthermore, the IL-4 and IL-13 receptors have been shown to be expressed on both HT-29 and Caco-2 cells (Trejdosiewicz *et al.*, 1998). Thus, the reason for the lack of regulation of induced COX-2 mRNA by IL-4 and IL-13 in Caco-2 cells remains surprising and unclear.

Finally, it has already been mentioned that IL-13 is known to activate PI 3-Kinase in HT-29 cells (Wright *et al.*, 1997). IL-4 has been shown to activate PI 3-Kinase in other systems and has similar immunoregulatory effects as IL-13 in intestinal systems, probably as a consequence of shared receptors and signalling pathways (Ahmad *et al.*, 1999; Mirmonsef *et al.*, 1999; Montaner *et al.*, 1999). This work therefore addressed whether the similar responses of HT-29 cells to IL-4 and IL-13 were reflected in similar activation of PI 3-Kinase. For this work, the

phosphorylation of PKB, a downstream effector of PI 3-Kinase, was used as a marker of its activation. This phosphorylation is generally regarded a being PI 3-Kinase dependent and is often used as a marker for PI 3-Kinase activation. In HT-29 cells IL-4 and IL-13, but not IL-10, resulted in a time dependent increase in the phosphorylation of PKB (Figure 5.9). In HT-29 cells there is known to be some constitutive PI 3-Kinase activity in keeping with their derivation from a human colorectal carcinoma. This explains the basal levels of PKB phosphorylation seen in the unstimulated samples. The similarity of response to IL-4 and IL-13 is unsurprising but represents the first description of IL-4 activating a PI 3-Kinase process, and by inference PI 3-Kinase, in this system. Again IL-10 caused no response in HT-29 cells.

Activation of PI 3-Kinase by IL-13 in this system has already been shown to be the mechanism by which IL-13 inhibits iNOS expression in HT-29 cells. COX-2 and iNOS share many characterisitics and the possibility that PI 3-Kinase may mediate the inhibitory action of IL-4 and IL-13 on COX-2 expression in HT-29 cells was explored in chapter 6. A variety of other mechanistic pathways having been proposed for the effects of Th2 cytokines on COX-2 expression (Niiro *et al.*, 1998; Diaz-Cazorla *et al.*, 1999; Paul *et al.*, 1999) but these are not necessarily mutually exclusive.

6 Results - Regulation of Induced COX-2 by PI 3-Kinase

6.1 Introduction

It has been shown in chapter 5 that the Th2 cytokines, IL-4 and IL-13, down-regulate cytokine induced COX-2 in HT-29 cells as well as activating the downstream effector of PI 3-Kinase, PKB. It is known that both of these cytokines can activate the PI 3-Kinase signalling pathway in a variety of cell systems (Mirmonsef *et al.*, 1999; Montaner *et al.*, 1999). Indeed it has been demonstrated that IL-13 can down-regulate iNOS in HT-29 cells and that this effect is mediated by this activation of PI 3-Kinase (Wright *et al.*, 1997). iNOS shares many biological characteristics with COX-2. It is induced in intestinal epithelial cells during inflammation (Singer *et al.*, 1996; Kolios *et al.*, 1998) and has been shown to be dependent on NFκB for its transcription in this cell system (Jobin *et al.*, 1998). This latter point links in with recent speculation concerning the findings that activation of NFκB is PI 3-Kinase dependent in some, but not all, systems (Ozes *et al.*, 1999; Madge and Pober, 2000). It was therefore investigated whether PI 3-Kinase had a regulatory role for cytokine induced, and thus NFκB dependent, COX-2 expression. Furthermore, recent work has revealed that PI 3-Kinase can be activated by TNFα directly (Hanna *et al.*, 1999; Kim *et al.*, 1999) and therefore the activation of PI 3-Kinase by TNFα was investigated in intestinal epithelial cells.

6.2 Results

6.2.1 COX-2 Expression and PI 3-Kinase Inhibitors

It was seen in chapter 4 that COX-2 expression can be induced in intestinal epithelial cells by both TNFα and IL-1α (see table 6.1 below).

	Induced COX-2	TNFα	IL-1α
HT-29	*mRNA*	✓	✓
	Protein	✓	✓
	PGE_2 product	✓	✗
Caco-2	*mRNA*	✗	✓
	Protein	✗	✓
	PGE_2 product	✗	✗

6.2.1.1 HT-29 cells and wortmannin

TNFα induced COX-2 in HT-29 cells was initially used to investigate the effect of the PI 3-Kinase inhibitor wortmannin. Pre-treatment with wortmannin, in the dark for 15 minutes, prior to the addition of TNFα resulted in markedly enhanced COX-2 mRNA expression in a concentration dependent manner (1-100nM) (Figure 6.1A). In the absence of cytokine stimulation, wortmannin had no effect (Figure 6.1B).

Having established that TNFα induced COX-2 mRNA expression is markedly up-regulated by wortmannin, it was next evaluated whether similar effects were observed at the level of protein expression. Indeed, wortmannin enhanced the TNFα stimulated COX-2 protein expression in a concentration dependent manner whilst having no effect on the constitutive isoform of COX, COX-1 (Figure 6.1C). Next, the effect of TNFα on COX-2 enzyme activity was investigated as assessed by production of one of its metabolic products, PGE_2. Wortmannin caused a significant concentration-dependent up-regulation of TNFα induced PGE_2 production correlating with the findings for mRNA and protein (Figure 6.1D).

To investigate whether the effects of PI 3-Kinase inhibition with wortmannin on induced COX-2 were unique to the TNFα stimulation, these experiments were repeated using IL-1α to induce COX-2. As with TNFα, HT-29 cells pre-treated with wortmannin for 15 minutes prior to the addition of IL-1α exhibited markedly enhanced COX-2 mRNA expression in a concentration dependent manner (0-100nM) (Figure 6.2A). A similar effect was seen with the potentiation of IL-1α induced COX-2 protein by wortmannin at 100nM (Figure 6.2B) but an effect on PGE_2 could not be assessed as IL-1α does not induce significant measurable PGE_2 production.

The up-regulation of cytokine induced COX-2 in HT-29 cells by wortmannin was seen at the level of COX-2 mRNA, as well as the downstream points of protein and product synthesis. In view of this, the effect of wortmannin on the stability of COX-2 mRNA was investigated to see whether this was the primary point of regulation. To assess this an Actinomycin D chase experiment was performed. HT-29 cells, in

the presence or absence of wortmannin pre-treatment (100nM for 15 minutes), were stimulated for two hours with either TNFα or IL-1α. At two hours Actinomycin D was added to give a concentration (5μg/ml) shown to inhibit all new mRNA synthesis in the system (Figure 5.2). mRNA was isolated at this point and at one hour and two hours thereafter and analysed for COX-2 mRNA by northern blot analysis. The decay of signal seen on northern blot analysis allows the calculation of a half life for COX-2 mRNA. This was also compared to TNFα or IL-1α stimulation alone where the cytokine containing media was washed off at two hours and replaced with Actinomycin D containing media. This was to check whether COX-2 mRNA stability was altered by the cytokines themselves, an effect which has been demonstrated in other systems (Huang *et al.*, 2000). For both TNFα and IL-1α stimulation, the half life of COX-2 mRNA was approximately one hour (Figure 6.3A and 6.3B). This was not significantly altered by washing off the stimulating cytokine at two hours implying that neither TNFα nor IL-1α alters COX-2 mRNA stability in HT-29 cells. However the presence of wortmannin caused a significant prolongation of COX-2 mRNA stability, induced by either TNFα ($p<0.05$) or IL-1α ($p<0.01$)(Figure 6.3A and 6.3B). This would explain the increased COX-2 mRNA seen in the presence of wortmannin and may contribute to the "downstream" increase in COX-2 product and PGE_2 product.

6.2.1.2 HT-29 cells and LY294002

Having demonstrated in HT-29 cells that PI 3-Kinase inhibition with wortmannin caused a stabilisation of COX-2 mRNA and increased COX-2 protein and PGE_2 product, the effect of a structurally distinct PI 3-Kinase inhibitor, LY294002, was

investigated. Pre-treatment of HT-29 cells with LY294002 for 30 minutes prior to the addition of TNFα resulted in potentiation of induced COX-2 mRNA at concentrations ≤1μM (Figure 6.4A). Above this concentration there was inhibition of COX-2 mRNA with LY294002 alone (1 and 3μM) having no effect on COX-2 mRNA (Figure 6.4A and 6.4B). To investigate this biphasic concentration response further, the effect of LY294002 on COX-2 protein was assessed. Western blot analysis showed a similar biphasic concentration response to LY294002 but there was no overall potentiation of COX-2 protein as was seen with COX-2 mRNA (Figure 6.4C). Indeed, LY294002 caused a partial inhibition of TNFα induced COX-2 protein at low (0.3μM) and high (10μM) concentrations. However, the inhibition of COX-2 protein seen at the highest concentration of 10μM was more complete than that observed with COX-2 mRNA. To pursue these findings the action of LY294002 on the COX-2 product PGE_2 was next quantified. This revealed a more linear significant concentration dependent inhibition of TNFα induced PGE_2, in direct conflict with the results seen with wortmannin (Figure 6.4D).

To see whether the observed the biphasic concentration response of TNFα induced COX-2 mRNA to LY294002 was dependent on the stimulating cytokine, the experiments were repeated with IL-1α as the inducing agent. This supported the findings with TNFα induction, showing potentiation of induced COX-2 mRNA by LY294002 at concentrations of ≤3μM with a peak at 0.3μM (Figure 6.5). There was similar inhibition of COX-2 mRNA at high inhibitor concentrations.

6.2.1.3 Caco-2 cells and PI 3-Kinase inhibitors

In order to support the potentially conflicting findings seen using two independent PI 3-Kinase inhibitors in HT-29 cells, similar experiments were carried out to investigate the actions of these inhibitors on the distinct intestinal epithelial cell line, Caco-2. Using IL-1α to induce COX-2 mRNA in these cells, the effect of both wortmannin and LY294002 pre-treatment was assessed. Wortmannin pre-treatment caused a concentration dependent up-regulation of induced COX-2 mRNA in a manner supporting the results seen in HT-29 cells (Figure 6.6A). For LY294002 a similar biphasic concentration response is seen on induced COX-2 mRNA in Caco-2 cells as was seen in HT-29 cells, although the regulatory changes are less marked (Figure 6.6B). There is minor potentiation of the COX-2 mRNA signal at 0.3μM and minor inhibition at the highest concentration used in this context, 3μM.

6.2.2 Activation of Phosphatidylinositol 3-Kinase by Th1 cytokines

To investigate whether Th1 cytokines can activate PI 3-Kinase in intestinal epithelial cells two independent sets of experiments were carried out. The first assessed the accumulation of PI 3-Kinase products, namely D-3 phosphoinositide lipids as measured by High Performance Liquid Chromatography (HPLC). Although any of the D-3 phosphoinositide lipids can be used as a marker for PI 3-Kinase activity, PtdIns(3,4)P_2 was chosen for use in this work as it was a major product which could be clearly and consistently identified allowing easy quantitative measurement (Figure 6.7). The second assessed PKB, a downstream effector of PI 3-Kinase, using its phosphorylation at Ser^{473} as a marker of it activation

6.2.2.1 TNFα induces the accumulation of D-3 phosphoinositide lipids

HT-29 cells were labelled with [^{32}P] and stimulated with TNFα (100ng/ml for 30 seconds) with or without wortmannin pre-treatment (100nm for 15 minutes in the dark). PI 3-Kinase activation was determined by measuring the accumulation of its products, the D-3 phosphoinositide lipids, by HPLC as seen on representative chromatograms (Figure 6.7). The HT-29 intestinal epithelial cell line is known to have some constitutive PI 3-Kinase activity which was reflected in a baseline amount of GroPtdIns(3,4)P_2 production in the blank control (Wright *et al.*, 1997)(Figure 6.8). Stimulation with TNFα resulted in a significant ($p<0.02$) increase of GroPtdIns(3,4)P_2 above basal levels, and this was inhibited by wortmannin (Figure 6.8).

6.2.2.2 Activation of PKB by Th1 cytokines

To support the observed activation of PI 3-Kinase by TNFα in HT-29 cells, the activation of a downstream effector of PI 3-Kinase, PKB, was assessed. This was done by western blot analysis using a specific antibody against the phospho-Ser^{473} within the PKB activation loop, which is only phosphorylated in activated PKB. HT-29 cells were treated in the presence or absence of wortmannin over a short time course (0-30 minutes). Protein was isolated, immunoprecipitated and probed for PKB Ser^{473} phosphorylation as described in Methods. The same blot was stripped and reprobed for pan PKB to demonstrate equal loading (Figure 6.9A). As would be expected there was some constitutive PKB activation in the control lane which was inhibited by the PI 3-Kinase inhibitor wortmannin. TNFα caused a time dependent

activation of PKB, peaking at 10 minutes, and this could be inhibited by wortmannin.

It was then assessed whether IL-1α could activate PKB in a PI 3-Kinase dependent manner. This has been reported in the literature in non-gastrointestinal systems but without the frequency of that seen with TNFα (Reddy *et al.*, 1997; Reddy *et al.*, 1998). IL-1α similarly activated PKB, although in a delayed manner with a peak at 20 minutes, and this was also inhibited by the PI 3-Kinase inhibitor wortmannin (Figure 6.9B).

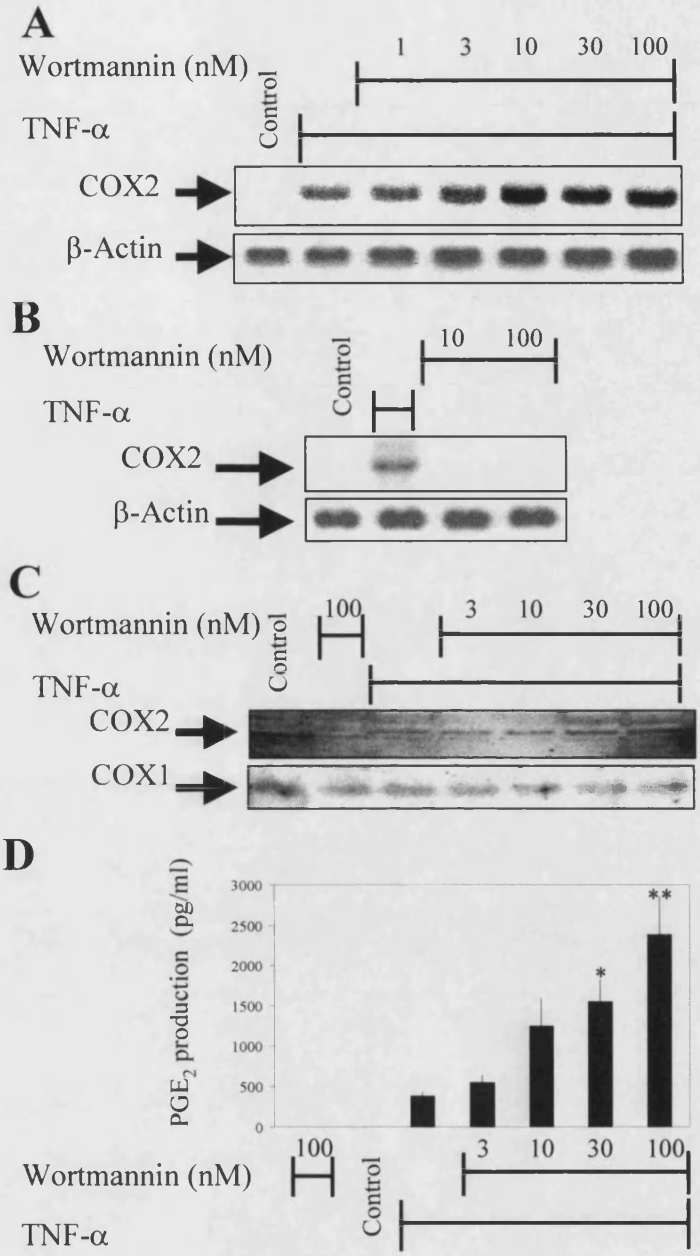


Figure 6.1: ***Up-regulation of TNFα induced COX2 expression by wortmannin.***
A and B. Northern analyses of mRNA isolated from HT-29 cells, which have been pre-treated with wortmannin (0-100nM) alone (**B**) or stimulated with TNFα (100ng/ml) for two hours (**A**) and probed for COX2 (upper panels). The membranes were stripped and reprobed for β-Actin to demonstrate loading (lower panels). **C**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with TNFα (100ng/ml) having been pre-treated with wortmannin (0-100nM), and probed with a specific anti-COX2 antibody (upper panel). The membrane was stripped and reprobed with a specific anti-COX1 antibody (lower panel). **D**. An ELISA for PGE_2 (pg/ml) using supernatants from HT-29 cells stimulated with TNFα (100ng/ml) for 24 hours having been pre-treated with wortmannin (0-100nM). Significant increases in PGE_2 production by additional wortmannin pre-treatment compared to TNFα alone are represented by * ($p<0.05$) and ** ($p<0.01$). This is the result of one experiment using triplicate samples and is representative of three other experiments. Blots are from single experiments but are representative of at least three others.

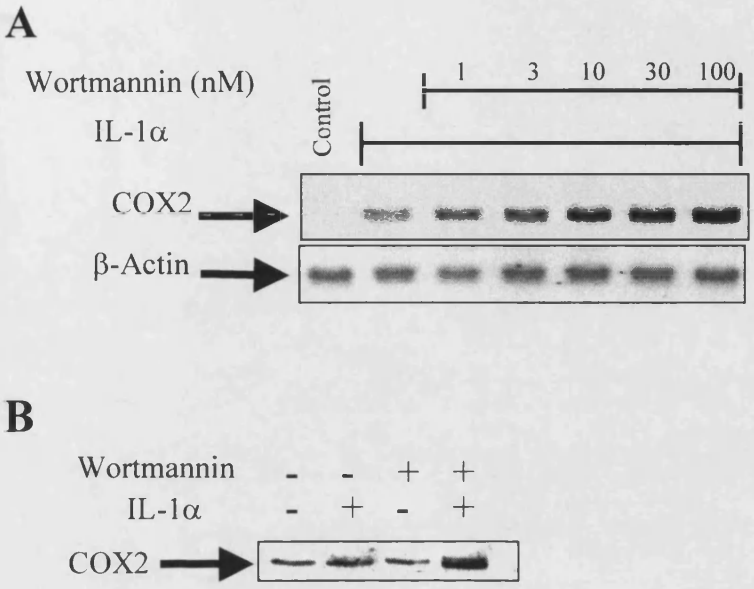


Figure 6.2: ***Up-regulation of IL-1α induced COX2 expression by wortmannin.*** **A**. Northern analysis of mRNA isolated from HT-29 cells, which have been stimulated with IL-1α (10ng/ml) for two hours having been pre-treated with increasing concentrations of wortmannin (0-100nM) and probed for COX2 (upper panel). The membrane was stripped and reprobed for β-Actin to demonstrate loading (lower panel). **B**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with IL-1α (10ng/ml) having again been pre-treated with wortmannin (100nM), and probed with a specific anti-COX2 antibody. Blots are from single experiments but are representative of at least three others.

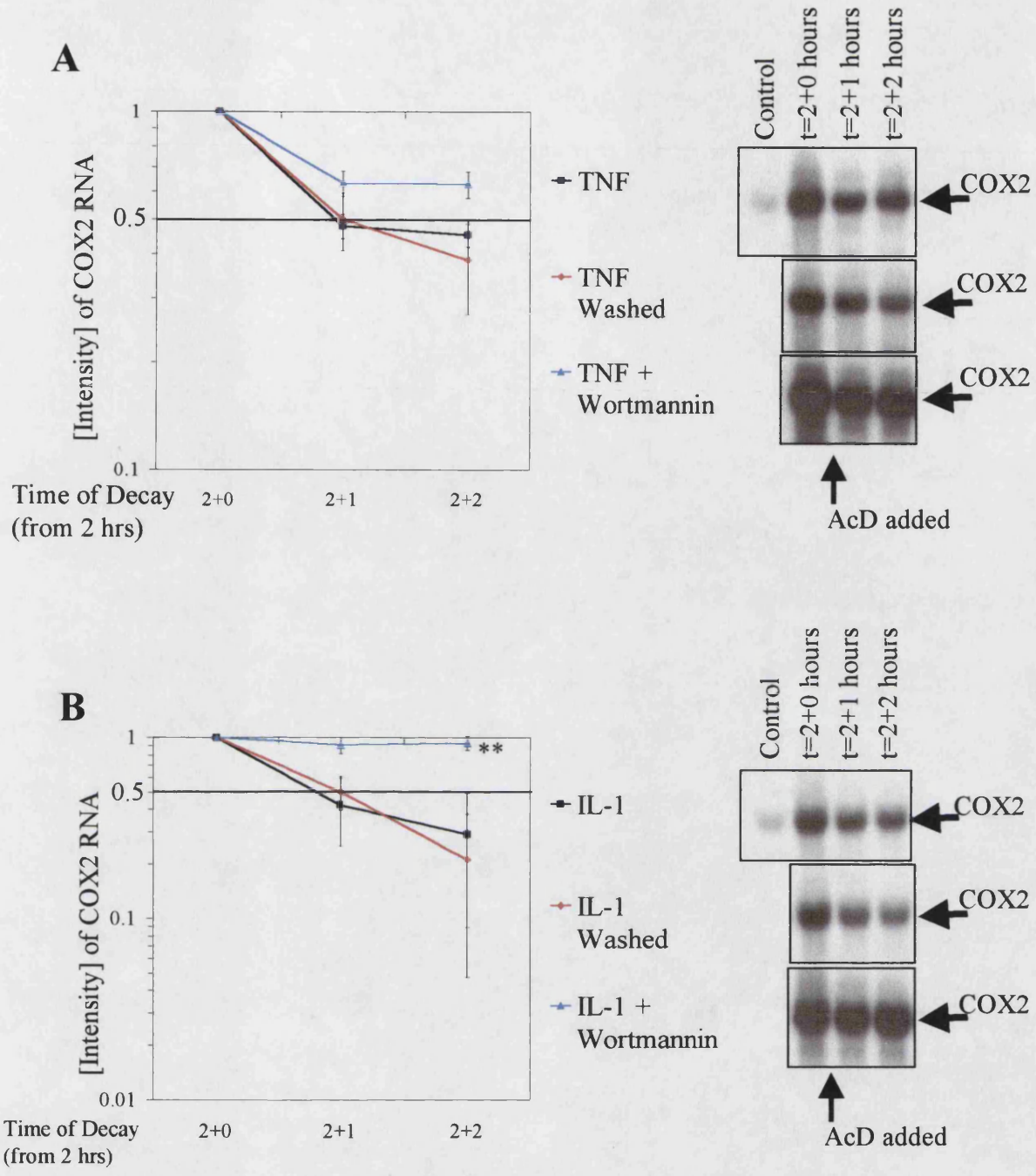


Figure 6.3: ***Stabilisation of COX2 mRNA by wortmannin***

Logarithmic line plots of [Intensity] of COX2 mRNA as measured by Northern analyses (arbitrary units where maximum intensity is 1) vs time in hours. HT-29 cells were stimulated with TNFα (**A**) or IL-1α (**B**) for 2 hours in the presence or absence of wortmannin (100nM). At 2 hours the stimulating cytokine was either washed off or remained in the media as labelled and Actinomycin D (5μg/ml) was added to prevent new RNA synthesis. The subsequent decay in the mRNA signal was plotted to allow calculation of the half life - time at which Intensity is 0.5. The blots shown are representative of two experiments. The membranes were photographed to show the 18S and 28S bands to ensure equal loading (not shown for clarity). * and ** represent significant ($p<0.05$ and $p<0.01$ respectively) prolongation of COX2 mRNA half life by the presence of wortmannin.

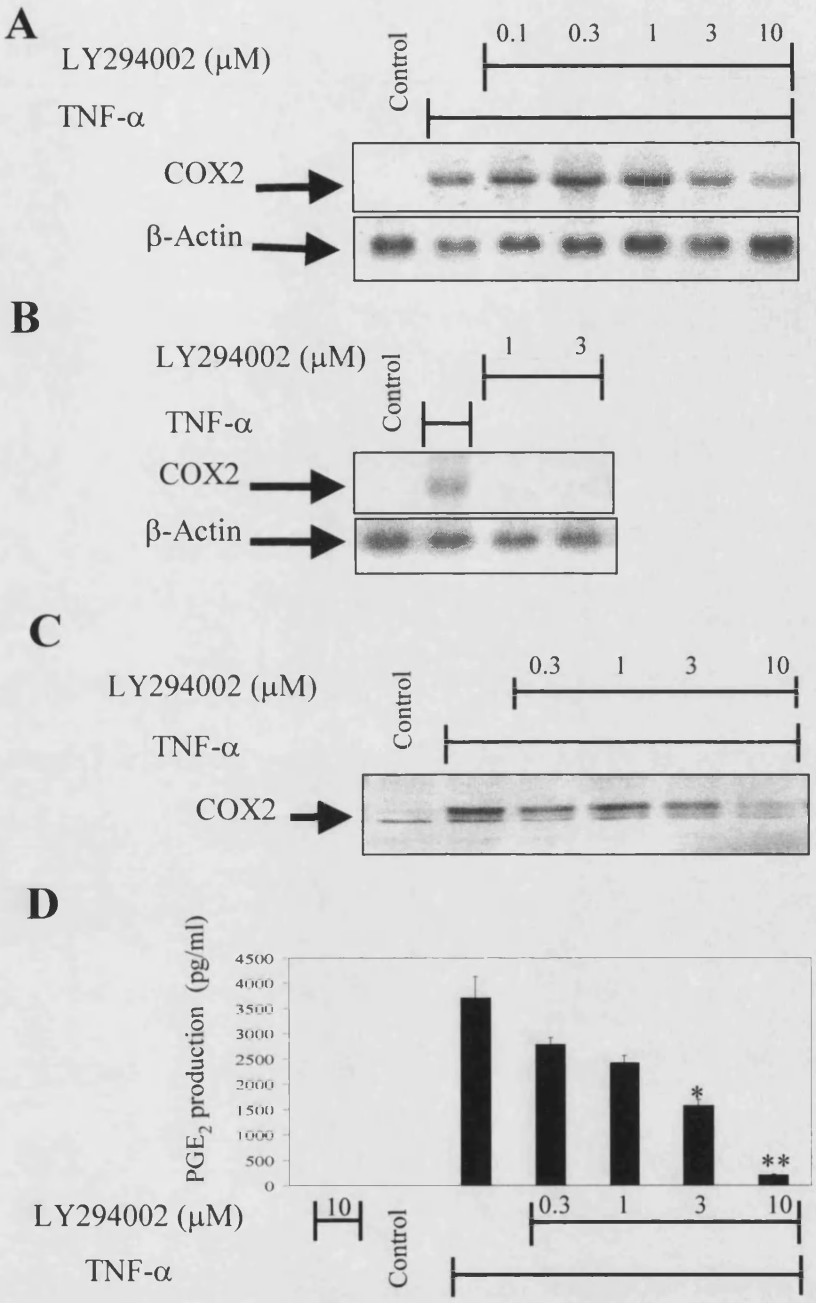


Figure 6.4: ***Regulation of TNFα induced COX2 expression by LY294002.***
A and B. Northern analyses of mRNA isolated from HT-29 cells, which have been pre-treated with LY294002 (0-10μM) alone (**B**) or stimulated with TNFα (100ng/ml) for two hours (**A**) and probed for COX2 (upper panels). The membranes were stripped and reprobed for β-Actin to demonstrate loading (lower panels). **C**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with TNFα (100ng/ml) having been pre-treated with LY294002 (0-10μM), and probed with a specific anti-COX2 antibody (upper panel). **D**. An ELISA for PGE_2 (pg/ml) using supernatants from HT-29 cells stimulated with TNFα (100ng/ml) for 48 hours having been pre-treated with LY294002 (0-10μM). * and ** represent significant ($p<0.05$ and $p<0.01$ respectively) inhibition of PGE_2 production by LY294002. This is the result of one experiment using triplicate samples and is representative of three other experiments. Blots are from single experiments but are representative of at least three others.

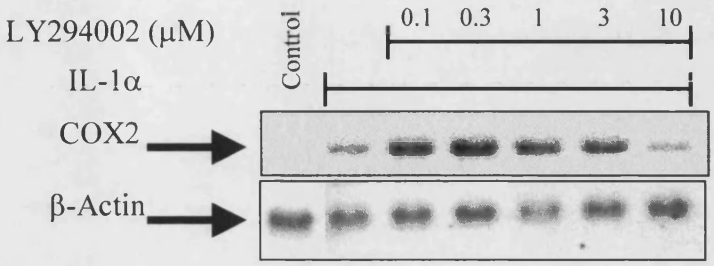


Figure 6.5: ***Regulation of IL-1α induced COX2 expression by LY294002.*** Northern analysis of mRNA isolated from HT-29 cells, which have been stimulated with IL-1α (10ng/ml) for two hours having been pre-treated with increasing concentrations of LY294002 (0-10μM) and probed for COX2 (upper panel). The membrane was stripped and reprobed for β-Actin to demonstrate loading (lower panel). Blots are from single experiments but are representative of at least three others.

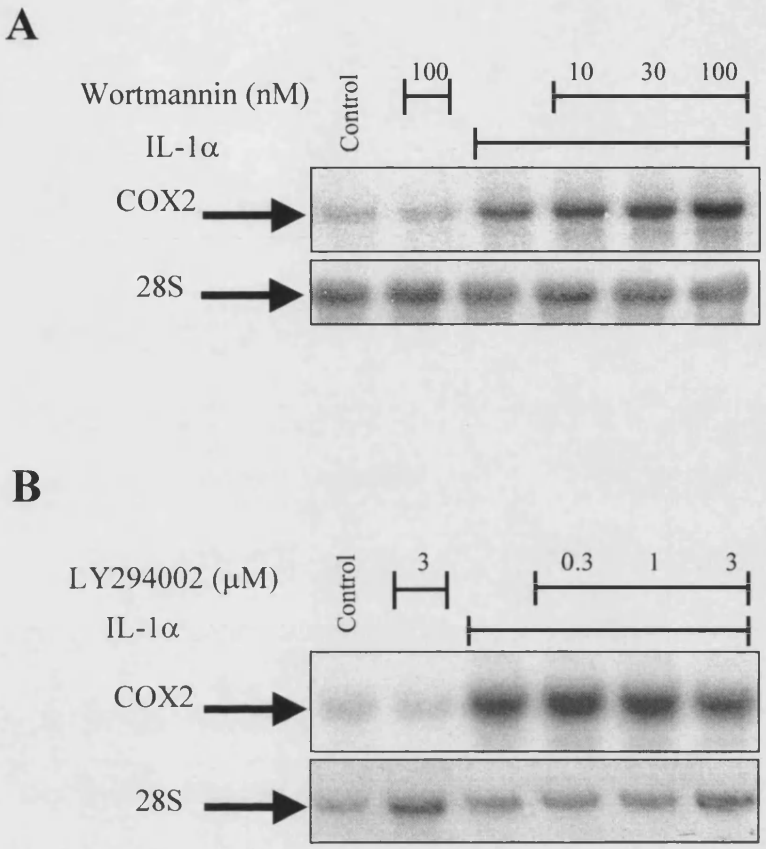


Figure 6.6: ***Regulation of IL-1α induced COX2 expression by PI 3-Kinase Inhibitors***. **A and B**. Northern analyses of mRNA isolated from Caco-2 cells, which have been stimulated with IL-1α (10ng/ml) for two hours having been pre-treated with increasing concentrations of wortmannin (0-100nM) (**A**) or LY294002 (0-10μM) (**B**) and probed for COX2 (upper panels). The membranes were stripped and reprobed for β-Actin to demonstrate loading (lower panels). Blots were from single experiments but are representative of at least three others.

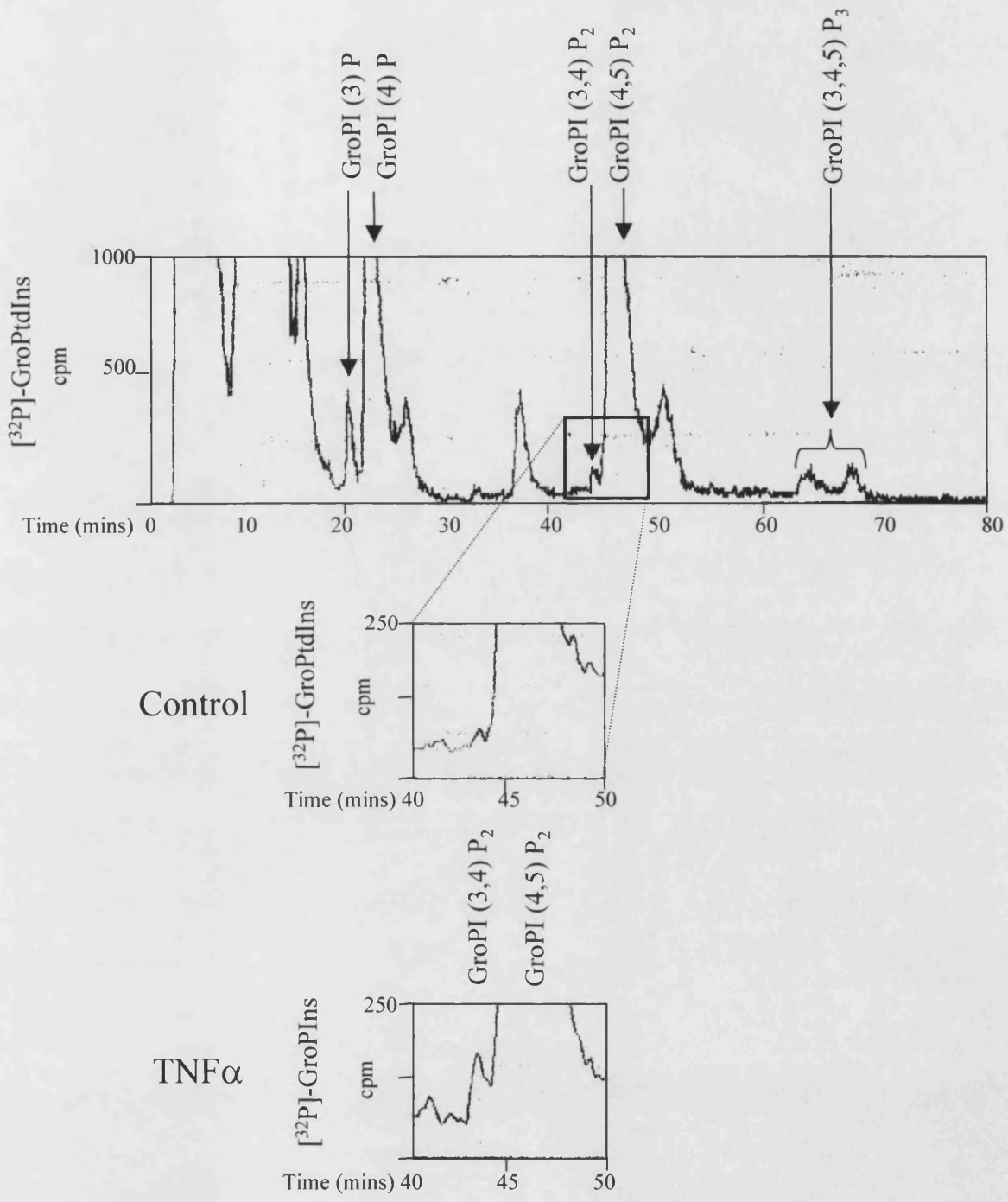


Figure 6.7: ***Activation of PI 3-Kinase by TNFα.***

A representative chromatogram trace, prior to analysis, showing the accumulation of the deacylated [^{32}P] labelled PI 3-Kinase product, GroPtdIns(3,4)P_2, as isolated by HPLC (cpm). [^{32}P] labelled HT-29 cells were stimulated with TNFα (100ng/ml) for 30 seconds. PtdIns lipids were then extracted and deacylated prior to HPLC separation. The control represents unstimulated cells. See Results Figure 6.8 for analysed results

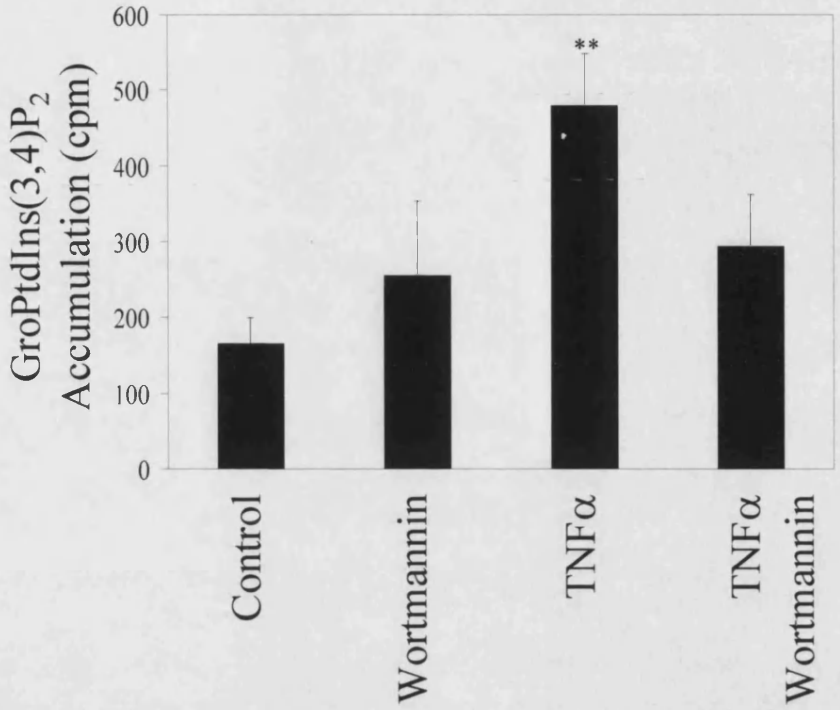


Figure 6.8: ***Activation of PI 3-Kinase by TNFα.***

Bar chart representing the accumulation of the [^{32}P] labelled PI 3-Kinase product, GroPtdIns(3,4)P_2, as isolated and quantified by HPLC (cpm). [^{32}P] labelled HT-29 cells were stimulated with TNFα (100ng/ml) for 30 seconds in the presence or absence of pre-treatment for 15 mins in the dark with the PI 3-Kinase inhibitor wortmannin (100nM). The controls represent unstimulated cells and cells treated with wortmannin alone. ** represents significant activation ($p<0.02$) of PI 3-Kinase by TNFα.

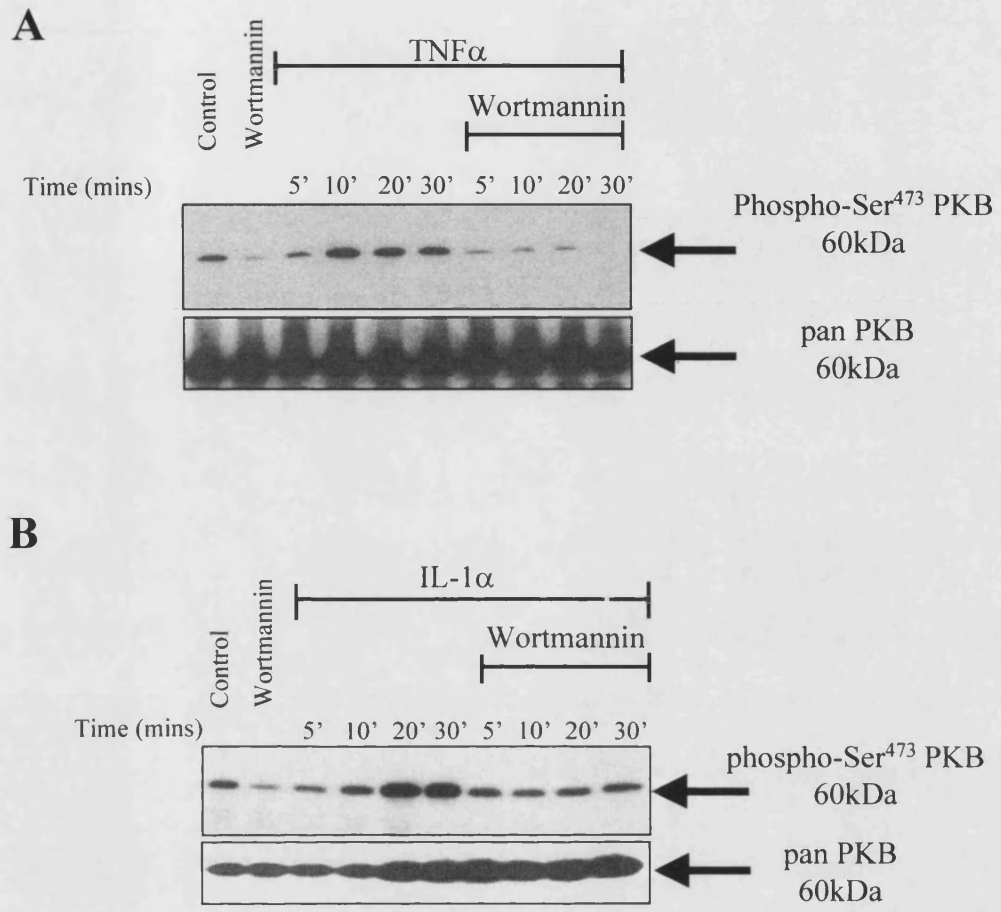


Figure 6.9: ***Activation of PKB by Th1 cytokines.***

A and B. Western analyses of protein isolated from HT-29 cells, stimulated over a short time course (0-30 mins) with TNFα (100ng/ml) (**A**) or IL-1α (10ng/ml) (**B**) in the presence or absence of wortmannin (100nM). Lysates were immunoprecipitated with specific goat anti-PKB antibody and then probed with a specific anti-phospho^{473} PKB antibody (upper panels). Membranes were stripped and reprobed for rabbit anti-PKB to demonstrate equal loading. Blots are from single experiments but are representative of two others.

6.3 Results Summary

- The PI 3-Kinase inhibitor wortmannin caused a concentration dependent up-regulation of induced COX-2. This was seen at the mRNA level in both HT-29 and Caco-2 cells, and at the level of COX-2 protein and PGE_2 product in HT-29 cells.
- It appears that wortmannin mediated this up-regulation of COX-2 mRNA, protein and product by stabilising the COX-2 mRNA and thereby significantly prolonging its half life.
- An alternative inhibitor of PI 3-Kinase, LY294002, also caused an up-regulation of induced COX-2 mRNA at low concentrations. However at higher concentrations LY294002 caused an inhibition of COX-2 mRNA.
- LY294002 did not potentiate induced COX-2 protein, indeed inhibiting COX-2 protein induction at low and high concentrations.
- LY294002 caused a significant concentration dependent inhibition of induced PGE_2 production.
- TNFα activated PI 3-Kinase in HT-29 cells as demonstrated by an accumulation of D-3 phosphoinositide lipids, and induction of phosphorylation and presumed activation of its downstream effector PKB. This activation of PI 3-Kinase was inhibited by wortmannin.
- IL-1α also caused the phosphorylation and presumed activation of the downstream effector of PI 3-Kinase, PKB in a wortmannin sensitive manner.

6.4 Discussion

The work presented here uses pharmacological inhibitors of PI 3-Kinase to provide evidence for a regulatory role for this signalling cascade on induced intestinal epithelial COX-2 expression. Such a role has already been demonstrated in this system with iNOS expression and activity as well as chemokine expression (Wright *et al.*, 1997; Kolios *et al.*, 1999). Indeed, in those contexts, PI 3-Kinase mediates the inhibitory effects of Th2 cytokines, such as IL-4 and IL-13, which were shown in chapter 5 to phosphorylate and presumably activate the downstream effector of PI 3-Kinase, PKB (Wright *et al.*, 1997). However the use of two pharmacological inhibitors of PI 3-Kinase, especially when they reveal at times contrasting and complex effects, necessitates further discussion.

Wortmannin is a PI 3-Kinase inhibitor derived from a metabolite of the fungus *Penicillium funiculosum Thom* (Wiesinger *et al.*, 1974; Park *et al.*, 1997). It covalently attaches to the p110 catalytic subunit and causes irreversible inhibition with a K_i of 1-10nM and a similar IC_{50}. In this work it caused a concentration dependent up-regulation of TNFα or IL-1α induced COX-2 mRNA in both HT-29 and Caco-2 cells, as well as of protein and PGE_2 production in HT-29 cells, at concentrations correlating well with its K_i.

This work also provides some evidence as to the mechanism of action of wortmannin. As its effects are seen at the point of mRNA assessment, it implies that it mediates its actions either by increasing transcription or increasing the stability of the induced COX-2 mRNA. The Actinomycin D chase experiments show that

incubation with wortmannin causes a significant stabilisation of COX-2 mRNA which would result in the marked increase seen on northern analysis. It is interesting to note that the continued presence or absence (by washing) of the stimulating cytokine does not alter COX-2 mRNA stability, as has been reported in other systems (Huang *et al.*, 2000). If PI 3-Kinase inhibition stabilises COX-2 mRNA, it implies that PI 3-Kinase pathways either act actively to destabilise COX-2 mRNA, or conversely prevent its stabilisation which is an important part of increasing COX-2 expression (Huang *et al.*, 2000). However, in this emerging area, the precise mechanisms for the effects of the PI 3-Kinase signalling cascade remain speculative. Taken overall, these results with wortmannin would strongly point to a negative regulatory role for PI 3-Kinase on induced intestinal epithelial COX-2 expression seen across two *in vitro* models. The evidence using a second pharmacological inhibitor of PI 3-Kinase is not completely supportive of this hypothesis.

LY294002 is a structurally distinct, competitive inhibitor for the adenosine triphosphate binding site of PI 3-Kinase and has been shown to cause specific inhibition with a K_i of 1.6μM and an IC_{50} of 1.4μM (Vlahos *et al.*, 1994). At concentrations of 1μM or less, LY294002 also had an up-regulatory effect on COX-2 mRNA expression in both HT-29 and Caco-2 cells. However at higher concentrations LY294002 inhibited TNFα and IL-1α induced COX-2 mRNA. Furthermore, it did not potentiate induced COX-2 protein in HT-29 cells, although it maintained a biphasic dose response curve with inhibition of induced COX-2 protein at low (0.3μM) and high (10μM) concentrations. Finally, LY294002 caused a significant concentration dependent inhibition of induced PGE_2 production in HT-29

cells in contrast to the action of wortmannin. There are a number of possible explanations of this difference between the two inhibitors.

Firstly, when regarding the actions of LY294002 on induced COX-2 mRNA in both HT-29 and Caco-2 cells, it can be seen that the initial potentiation occurred at concentrations consistent with the K_i and IC_{50}. The inhibition of induced COX-2 mRNA seen at higher concentrations may be due to non-specific actions of LY294002 at concentrations above those needed to inhibit PI 3-Kinase. Against this, however, are the results seen at the level of COX-2 protein and PGE_2 production.

A second possible explanation of the different response profiles for the two inhibitors may be as a result of disparate effects on different classes of the PI 3-Kinase family of enzymes. For example, wortmannin is a poor inhibitor of Class II PI 3-Kinases (Domin *et al.*, 1997). Such effects of specific classes may well be important in HT-29 cells where differential class effects of PI 3-Kinase enzymes are involved in controlling macroautophagy (Petiot *et al.*, 2000) and this may also explain why wortmannin only partially inhibits the TNFα induction of PI 3-Kinase (Figure 6.8).

Thirdly, wortmannin and LY294002 may not be as specific at inhibiting PI 3-Kinase as they claim. Although there are reports demonstrating the specificity of their actions (Wiesinger *et al.*, 1974; Vlahos *et al.*, 1994; Davies *et al.*, 2000), there are isolated reports suggesting other actions of these pharmacological compounds. Wortmannin has been reported as inhibiting phospholipase A_2 (Cross *et al.*, 1995),

although if this resulted in less arachidonic acid as substrate for COX-2 one might expect inhibition of PGE_2 production rather than potentiation. Reports that wortmannin can inhibit PI 4-Kinase (Nakanishi *et al.*, 1995) are more difficult to interpret, as is the finding that LY294002 can inhibit casein kinase (Davies *et al.*, 2000).

Finally, although disparity between results obtained using wortmannin and LY294002 are rare, there is a precedent (Salh *et al.*, 1998). This group were studying PI 3-Kinase and iNOS activation in a macrophage cell line and showed that activation of PI 3-Kinase by LPS was sensitive to both wortmannin and LY294002. However, nitric oxide generation was sensitive only to LY294002 and not to wortmannin. They proposed an FKBP12-rapamycin-associated protein (FRAP) dependent pathway which was more sensitive to LY294002 than to wortmannin. Supporting their proposition is evidence from a second group demonstrating that both wortmannin and LY294002 can inhibit FRAP at higher concentrations than those necessary to inhibit PI 3-Kinase (Brunn *et al.*, 1996).

To summarise the use of these pharmacological inhibitors of PI 3-Kinase, one must be aware of the potential pitfalls regarding the interpretation of the results. There are other methods of inhibiting PI 3-Kinase such as molecular methods like micro-injection of antibodies or transferring dominant negative forms of PI 3-Kinase into cells (Benistant *et al.*, 2000; Sasaki *et al.*, 2000; Stein and Waterfield, 2000; Weaver and Ward, 2001). Similar transfer methods can be used to manipulate PI 3-Kinase by causing its over-expression, and these may be placed under the control of an environmental agent such as an antibiotic in the culture media (Craddock *et al.*,

1999). Evidence should be gathered from different models using different mechanisms of manipulating PI 3-Kinase and from the resulting evidence a consensus should be drawn.

With the knowledge that Th2 cytokines activate PI 3-Kinase and inhibit COX-2 (chapter 5), supported by the consistent results with wortmannin, there is evidence supporting the hypothesis that PI 3-Kinase activity inhibits COX-2 expression in intestinal epithelial cells. Indeed, one interpretation of the results presented here is that basal PI 3-Kinase activity known to be present in HT-29 cells may be regulating COX-2 expression. Against this, however, is the fact that neither PI 3-Kinase inhibitor used here had any effect on COX-2 mRNA or protein expression or on PGE_2 production when used in the absence of cytokine stimulation. The results with LY294002 do bring an inhibitory role of PI 3-Kinase on COX-2 in to question. So too does work by Shao *et al* using tranfer of constitutively active or dominantly negative forms of PKB, the downstream effector of PI 3-Kinase, into HCA-7 and LS-174 human intestinal epithelial cells (Shao *et al.*, 2000). This work can be summarised as indicating a role for PKB in stabilising a COX-2 3′-UTR construct. This is at odds with the results presented here, but used a different method of inhibiting PI 3-Kinase as well as looking at a marker of COX-2 mRNA stability rather than its actual stability as used here.

The possibility that PI 3-Kinase, a signalling cascade with a known pro-proliferative action, could be inhibiting COX-2, which is also known to promote carcinogenesis, is surprising. However, PI 3-Kinases are a complex family of enzymes which may

have conflicting actions. Also, more evidence is needed to confirm the exact inter-relationship between PI 3-Kinases and COX-2 expression.

One aspect of conformity involving the use of these inhibitors should be commented on. There have been reports in epithelial cells that the TNFα induced activation of NFκB, known to be crucial to COX-2 expression, is dependent on PI 3-Kinase activation (Ozes *et al.*, 1999; Reddy *et al.*, 2000). Work by collaborating colleagues in University of North Carolina – Chapel Hill demonstrated that the activation of NFκB by TNFα or IL-1 was not affected by incubation with either of the inhibitors of PI 3-Kinase (Appendix A) (Weaver *et al.*, 2001). This implies that any effect seen using either PI 3-Kinase inhibitor does not appear to be mediated via alteration of NFκB activation. It also implies that activation of NFκB is not PI 3-Kinase dependent in this system, something which has been shown in endothelial cells (Madge and Pober, 2000). Other investigators have shown that PI 3-Kinase inhibition does alter NFκB activity in this system, but over a much longer time period and with a much higher inhibitor concentration (Wang *et al.*, 2000).

The next subject addressed in this chapter was whether Th1 cytokines could activate PI 3-Kinase directly. This follows on from the work mentioned above concerning TNFα activation of NFκB being PI 3-Kinase dependent in some systems. Recent research has shown that TNFα can activate PI 3-Kinase in HeLa cells (Pastorino *et al.*, 1999), rat fibroblasts (Hanna *et al.*, 1999), cervical epithelial cells (Ozes *et al.*, 1999) and embryonic kidney cells (Ozes *et al.*, 1999), although the biological implications of this are uncertain. We have also shown that TNFα does activate PI 3-Kinase directly, beyond the constitutive activity that these cells are known to

demonstrate, as well as inducing the phosphorylation and presumed activation of the downstream effector of PI 3-Kinase, PKB. Furthermore, the other Th1 cytokine shown to induce COX-2 in HT-29 cells, IL-1α, also induces the phosphorylation of PKB implying activation of PI 3-Kinase as has been seen in other systems (Donaldson *et al.*, 1996; Reddy *et al.*, 1997; Madge and Pober, 2000).

In intestinal epithelial cells TNFα is now known to activate pro-apoptotic pathways and the NFκB signaling pathway as well as the pro-proliferative PI 3-Kinase as demonstrated here (Papadakis and Targan, 2000). Making sense of these conflicting intracellular signals highlights the multiple controls of cellular responses with the overall outcome representing the final balance of the divergent pathways.

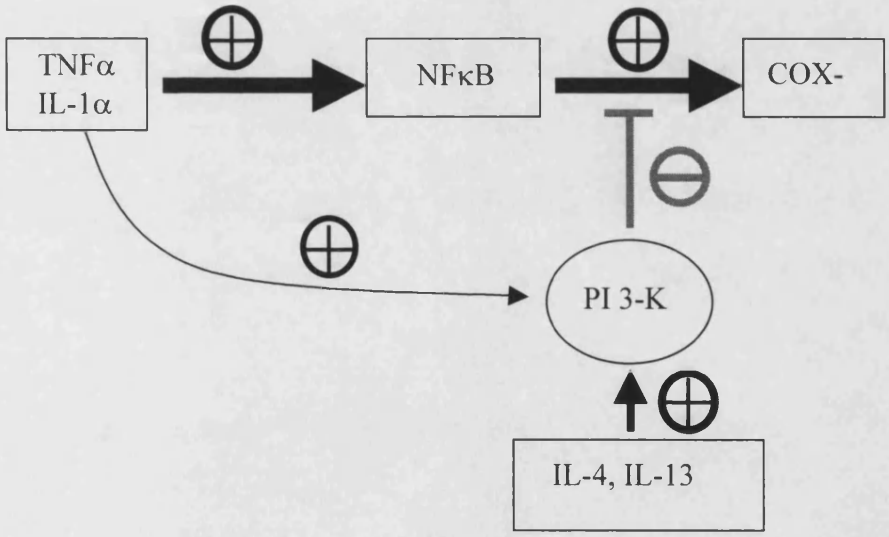


Figure 7A: ***Schematic representation of the possible involvement of PI 3-Kinase in the regulation of cytokine induced COX-2***

If PI 3-Kinase does inhibit COX-2 expression and mediate the inhibitory action of Th2 cytokines, as much of the evidence presented here supports, then the TNFα activation of PI 3-Kinase will act as a negative autoregulatory pathway on the TNFα

activation of NFκB dependent COX-2 expression. Such autoregulatory feedback loops are likely to be repeated many times in the cell with the apparent redundancy of mechanisms allowing better controlled and smoother cellular responses (see Figure 7A).

7 Mitogen Activated Protein Kinases – their activation, interactions and role in COX-2 expression

7.1 Introduction

The Mitogen Activated Protein Kinases (MAP Kinases) represent a family of signalling pathways which are important in the propagation of inflammation and developmental regulation (Tibbles and Woodgett, 1999). In some systems they are known to regulate COX-2 transcription and mRNA stability (Dean *et al.*, 1999), and are also known to mediate the regulatory effects of cytokines on COX-2 expression (Niiro *et al.*, 1998). Indeed, there is increasing evidence that they are involved in "cross-talk" with other signalling pathways known to be important in COX-2 regulation, namely NFκB and PI 3-Kinase (Yamaki *et al.*, 2000). Therefore, this work aimed to address whether MAP Kinases were activated by the pro-inflammatory cytokines TNFα and IL-1α which have already been shown to induce COX-2, activate PI 3-Kinase and known to activate NFκB. Secondly, specific inhibitors of MAP Kinases were used to investigate whether MAP Kinases had a regulatory role for COX-2 in this gastrointestinal system. Thirdly, the issue of MAP Kinase activation being mediated by PI 3-Kinases was addressed. Finally, the inactivation by phosphorylation of a downstream substrate of PKB, Glycogen Synthase Kinase 3α/β, was assessed to determine the relative contributions of PI 3-Kinase and ERK1/2 and possible synergistic action of these two signalling pathways.

7.2 Results

7.2.1 MAP Kinase activation

7.2.1.1 TNFα and MAP Kinase activation

To investigate activation of MAP Kinases in intestinal epithelial cells TNFα was first assessed since it is known to activate NFκB and PI 3-Kinase in HT-29 cells. Confluent monolayers of HT-29 cells were stimulated over a short time course of up to thirty minutes with TNFα at 100ng/ml and then protein was isolated for western blot analysis. Whole cell lysates were run on western blots and probed with specific rabbit antibodies against the phosphorylated (activated) MAP Kinases – phospho$^{180, 182}$ p38, phospho$^{183, 185}$ JNK or phospho$^{202, 204}$ ERK1/2. Membranes were then stripped and reprobed for the respective un-phosphorylated isoforms (Figure 7.1). TNFα was shown to activate p38 and JNK transiently, whilst ERK activation was more sustained. All three MAP Kinases were optimally phosphorylated at 10 minutes.

7.2.1.2 IL-1α and MAP Kinase activation

Having demonstrated that TNFα activates p38, JNK and ERK1/2 MAP Kinases in HT-29 intestinal epithelial cells, activation by IL-1α was next investigated. Using similar experimental protocols, HT-29 cells were stimulated with IL-1α (10ng/ml) over a short time course up to 30 minutes. Protein was isolated for western blot

analysis as previously, probing for the activated forms of p38, JNK and ERK1/2 showed IL-1α also activated these MAP Kinases (Figure 7.2). This was qualitatively different from MAP Kinase activation by TNFα as the activation was still increasing at 30 minutes rather than peaking at 10 minutes. Stripping and reprobing the membranes, with the respective un-phosphorylated isoforms, demonstrated equal loading (Figure 7.2).

7.2.1.3 ERK1/2 and p38 activation in the presence of their specific inhibitors

Understanding of the role of MAP Kinases in cell physiology has been improved by the growing use of specific inhibitors. A variety of specific inhibitors exist for the ERK1/2 and the p38 pathways although not for the JNK pathway. It was planned to use inhibitors of ERK1/2 and p38, PD98059 and SB203580 respectively, to investigate the role of these signalling components in COX-2 expression. Prior to doing this, these compounds were first used to determine their effect on activation of their respective MAP Kinase pathways.

Firstly, the effect of PD98059 on ERK1/2 was determined since PD98059 is known to inhibit the upstream kinases, MEK1/2, which control ERK1/2 phosphorylation. HT-29 cells were stimulated with TNFα over a short time course of 30 minutes, in the presence or absence of pre-treatment with PD98059 at 10μM for one hour. Protein was isolated and analysed by western blot analysis with a specific antibody against the active form of ERK1/2 (Figure 7.3). The presence of PD98059 caused a significant but not complete abrogation in ERK1/2 phosphorylation when compared

to TNFα alone. Stripping and reprobing the membranes, with the respective un-phosphorylated isoforms, demonstrated equal loading (Figure 7.3).

The situation with the selected p38 inhibitor, SB203580, proved to be more complex. This is a reversible inhibitor of the ATP binding site of p38 with conflicting evidence regarding its action on activation of p38 by phosphorylation (Young *et al.*, 1997). HT-29 cells were stimulated over a short time course of 30 minutes with TNFα in the presence or absence of pre-treatment with SB203580 at 10μM for one hour. Protein was isolated and analysed by western blot analysis with a specific antibody against the active form of p38 (Figure 7.4A). The presence of SB203580 had little effect on phosphorylation status, and by inference activation, of p38 when compared to TNFα alone. Stripping and reprobing the membranes, with the respective un-phosphorylated isoforms, demonstrated equal loading (Figure 7.4A).

In view of the fact that there is evidence that SB203580 effectively binding the ATP site may not alter the phosphorylation of p38, an *in vitro* kinase assay for p38 activity was next performed. HT-29 cells were stimulated as previously with TNFα in the presence or absence of pre-treatment with SB203580. Protein was isolated and the p38 kinase assay was performed as described in methods. The functional readout of this assay was p38 dependent *in vitro* phosphorylation of a known p38 substrate, namely the transcription factor ATF-2 (Figure 7.4B). It can be seen that the presence of SB203580 did not alter the TNFα stimulated p38 activity, as assessed by ATF-2 phosphorylation. TNFα stimulated p38 activity peaked at 20 minutes, approximately 10 minutes after peak p38 phosphorylation seen in the

associated blot of whole cell lysate. Stripping and reprobing for unphosphorylated p38 demonstrated equal loading.

This lack of inhibition of p38 activity by SB203580 may be due to the reversible nature of the inhibitor and it being washed off in the immunoprecipitation steps of the kinase assay. In this case it should be possible to reintroduce SB203580 after the washing steps and see an inhibition of p38 activity. Such data has been discussed but not shown in the literature (Young *et al.*, 1997). However, an attempt to reintroduce SB203580 to the kinase assay for 15 minutes after the washing steps failed to demonstrate significant inhibition of p38 kinase activity (Figure 7.4C).

7.2.2 MAP Kinase inhibition and COX-2 expression

7.2.2.1 PD98059 and COX-2 expression

Having demonstrated that the specific MEK1/2 inhibitor does inhibit activation of ERK1/2, this compound was then used to assess whether ERK1/2 activation had a role in the induction of COX-2 expression. HT-29 cells were stimulated with TNFα in the presence or absence of PD98059 (0-30μM) for 2 hours and then RNA was isolated and probed for COX-2 mRNA by Northern blot analysis (Figure 7.5A). Surprisingly, PD98059 caused an increase in stimulated COX-2 mRNA production at concentrations of 10 and 30μM.

In light of the knowledge that there can be opposing regulatory effects at different points in the RNA to protein to product pathway, as seen with IFNγ (chapter 5), the

effect of ERK1/2 inhibition was investigated at the level of COX-2 protein and product expression. Protein isolated from HT-29 cells pre-treated with PD98059 and then stimulated with TNFα for six hours, showed that COX-2 protein is partially inhibited at higher concentrations (10μM) of this inhibitor (Figure 7.5B). When looking at cells stimulated for 72 hours, and where supernatants were collected for PGE_2 assay, PD98059 caused a significant concentration dependent inhibition of PGE_2 generation with an IC_{50} of approximately 1.5μM (Figure 7.5C). This is a far lower concentration than those causing the effects on COX-2 mRNA and protein.

7.2.2.2 SB203580 and COX-2 expression

Having shown that ERK1/2 appear to play a part in inducing COX-2 expression in HT-29 cells, a similar range of experiments were planned using the p38 inhibitor SB203580. As seen previously, this compound did not inhibit p38 phosphorylation or indeed activity in the context of an *in vitro* p38 kinase assay. However, there were possible molecular and methodological reasons for this and the action of this compound, which is widely used as a p38 inhibitor in the literature, was explored on COX-2 mRNA and protein expression as well as PGE_2 production.

HT-29 cells were stimulated as previously with TNFα for two hours, in the presence or absence of one hour pre-treatment with SB203580 at 0-30μM, with COX-2 mRNA expression being assessed by Northern blot analysis (Figure 7.6A). p38 inhibition caused a slight decrease of COX-2 mRNA expression at high concentrations (10 and 30μM). A similar effect of p38 inhibition was seen at slightly lower concentrations on COX-2 protein induced by TNFα stimulation for six

hours (Figure 7.6B). At 3 and 10μM there was slight inhibition of COX-2 protein induction. Finally, SB203580 was seen to have a similar significant inhibitory action to PD98059 on TNFα induced PGE_2 production, at similar concentrations (IC_{50} approximately 1.5μM).

7.2.2.3 Combined p38 and ERK1/2 inhibition on COX-2 expression

The demonstration of both p38 and ERK1/2 inhibition individually causing a concentration dependent inhibition to COX-2 functional activity raised speculation that there may be a greater effect if used in combination as has been shown in other systems (LaPointe and Isenovic, 1999; Rutault *et al.*, 2001). This was explored in HT-29 intestinal epithelial cells at the level of TNFα induced COX-2 protein expression and PGE_2 production. As is seen in Figure 7.7A, combined pre-treatment with both inhibitors at 3μM did not cause a greater inhibition of COX-2 protein at six hours as seen by western blot, with there being no alteration of the constitutive isoform of COX, COX-1. Similarly, TNFα induced PGE_2 production by HT-29 cells at 72 hours was inhibited by both compounds as previously described, but there was no additive effect of the inhibitors used in combination (Figure 7.7B).

7.2.3 MAP Kinase activation in the presence of PI 3-Kinase inhibition

7.2.3.1 MAP Kinase activation in the presence of wortmannin

It has been previously shown in chapter 6 that the PI 3-Kinase inhibitor wortmannin caused a concentration dependent up-regulation of induced COX-2 mRNA and

protein expression as well as PGE_2 production. In combination with the findings regarding the role of p38 and ERK1/2 in COX-2 expression, this led to the possibility that wortmannin may be exerting its effects by promoting MAP Kinase activation. In contrast, published literature reports that the activation of MAP Kinases may be PI 3-Kinase dependent, although this has not been shown in a gastro-intestinal system (Yamaki *et al.*, 2000; Jiang *et al.*, 2000; Yamboliev *et al.*, 2000).

The previously described activation of p38, ERK1/2 and JNK by TNFα was therefore assessed in the presence or absence of wortmannin pre-treatment at 100nM for 15 minutes. Figure 7.8 shows this comparison with it being seen that the addition of wortmannin pre-treatment does not alter TNFα induced phosphorylation of p38, ERK1/2 or JNK.

7.2.3.2 Cytokine induced GSK-3α/β inactivation

Glycogen Synthase Kinase-3α/β (GSK-3α/β) is an example of an enzyme which is known to be phosphorylated by PI 3-Kinase dependent mechanisms (Cross *et al.*, 1995; Stein and Waterfield, 2000). GSK-3α/β is a constitutively active enzyme that is inactivated by phosphorylation. HT-29 cells were incubated with TNFα or IL-1α over a 30 minute time course, protein was isolated for western blot analysis and the membranes were probed for GSK-3α/β. Figure 7.9 shows that IL-1α and TNFα can both induce the phosphorylation and therefore presumably inactivation of GSK-3α/β in a time dependent fashion. IL-13 stimulation for 10 minutes, as a known activator of PI 3-Kinase, is shown as a known positive control, which is abrogated

by wortmannin pre-treatment (100nM for 15 minutes). As with MAP Kinases, IL-1α induced phosphorylation occurs later than that seen with TNFα, which peaks at 10 minutes.

HT-29 cells were then stimulated with TNFα over a 30 minute time course, having been pre-treated with wortmannin at 100nM for one hour, and the subsequently generated western membranes were probed for phospho-GSK-3α/β. Wortmannin is shown to partially abrogate the TNFα induced phosphorylation of GSK-3α/β (Figure 7.10A) as would be expected given the documented involvement of PI 3-Kinase in this pathway. However, as the effect of wortmannin was only partial abrogation, it was combined with the MEK1/2 inhibitor of the ERK1/2 pathway, PD98059. With this combination of wortmannin (100nM) and PD98059 (10μM), there is complete inhibition of the TNFα induced phosphorylation of GSK-3α/β (Figure 7.10B). PD98059 alone, like wortmannin, had a partial effect. This clearly demonstrates that the reagents used were active, and that there is a role for activation of both ERK1/2 and PI 3-Kinase pathways in the phosphorylation of GSK-3α/β in HT-29 cells.

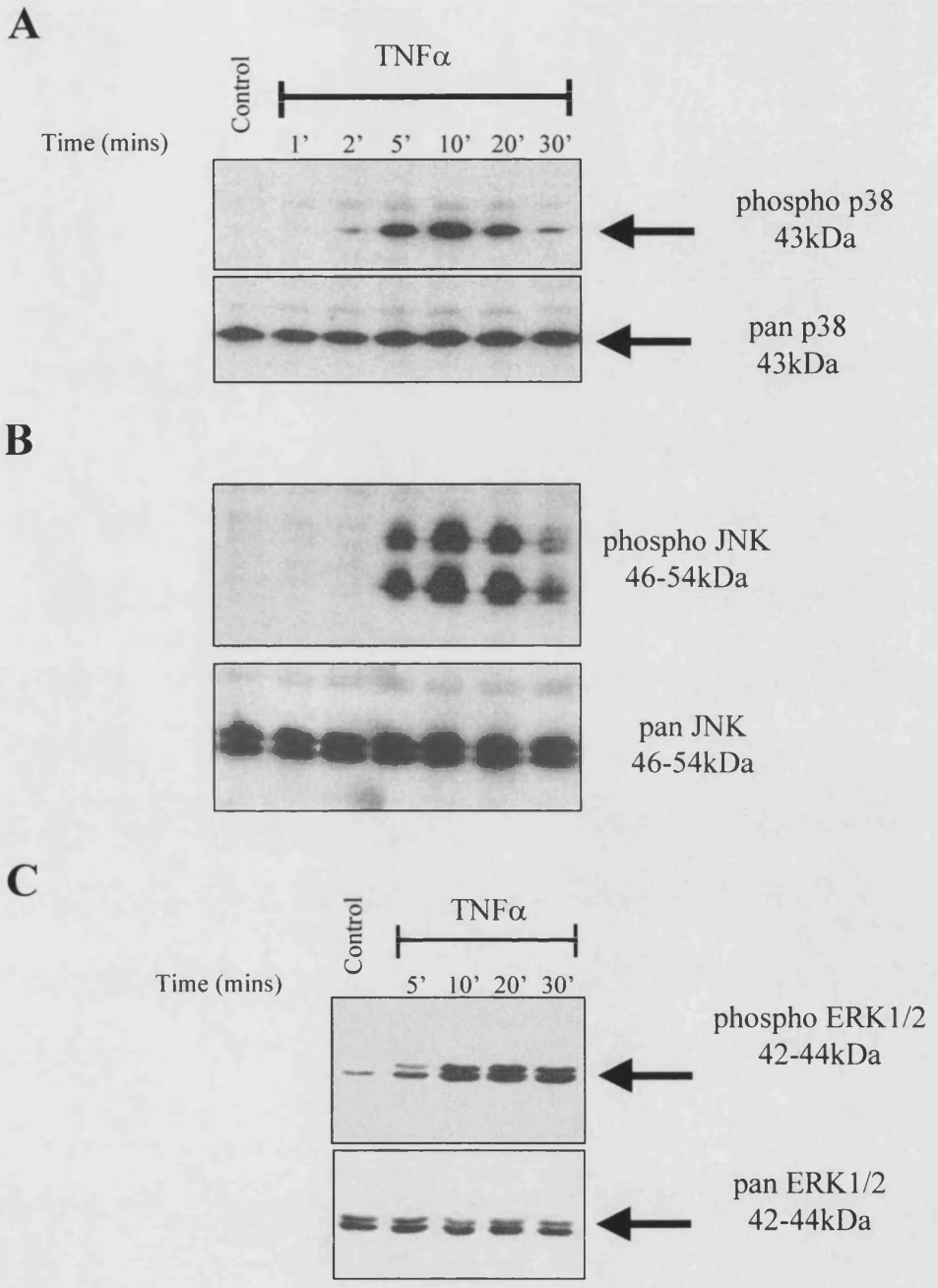


Figure 7.1: ***Activation of MAP Kinases by TNFα in HT-29 cells.***
A to C. Western analyses of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course and then probed with specific antibodies against: phospho$^{180, 182}$ p38 (**A**), phospho$^{183, 185}$ JNK (**B**) or phospho$^{202, 204}$ ERK1/2 (**C**) (upper panels). Membranes were then stripped and reprobed with the respective antibodies against the unphosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least three others.

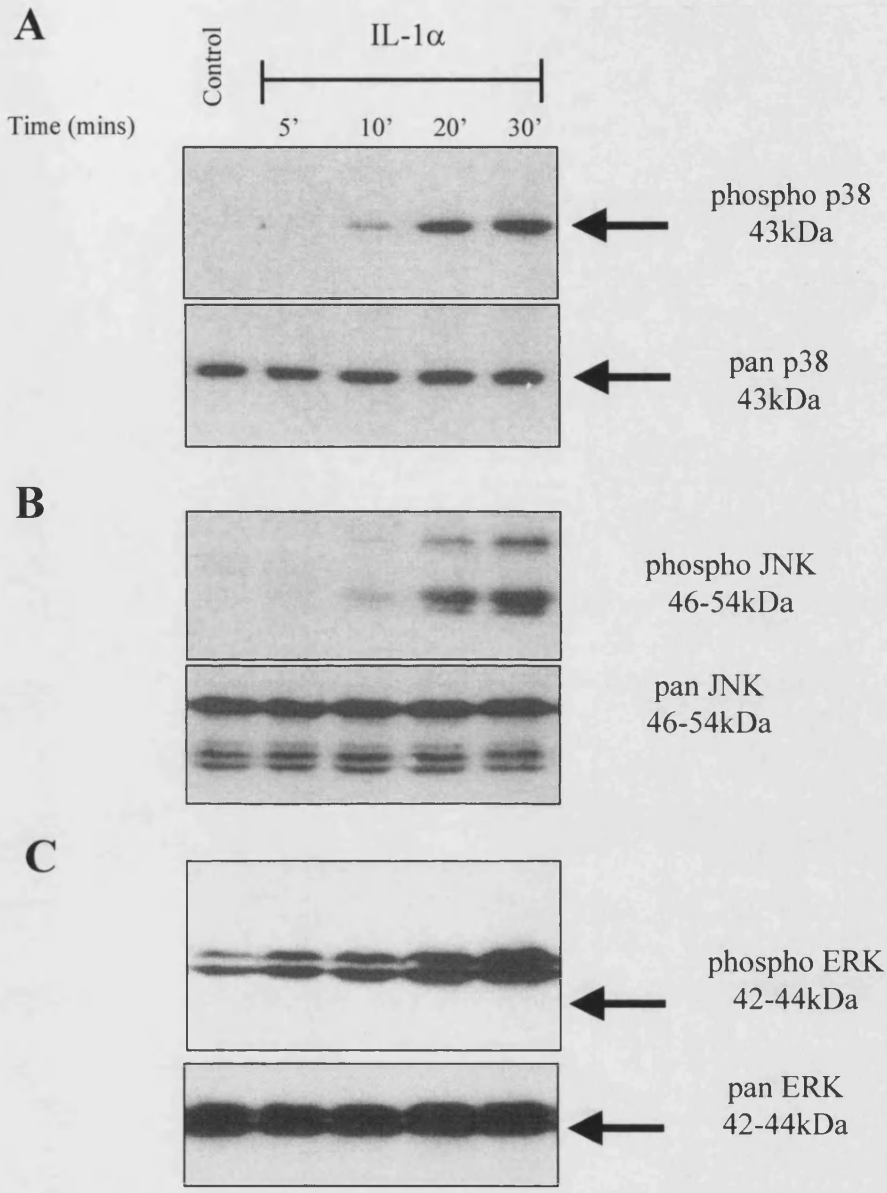


Figure 7.2: ***Activation of MAP Kinases by IL-1α in HT-29 cells.***
A to C. Western analyses of protein isolated from HT-29 cells stimulated with IL-1α (10ng/ml) over a 30 minute time course and then probed with specific antibodies against: phospho$^{180, 182}$ p38 (**A**), phospho$^{183, 185}$ JNK (**B**) or phospho$^{202, 204}$ ERK1/2 (**C**) (upper panels). Membranes were then stripped and reprobed with the respective antibodies against the unphosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least three others.

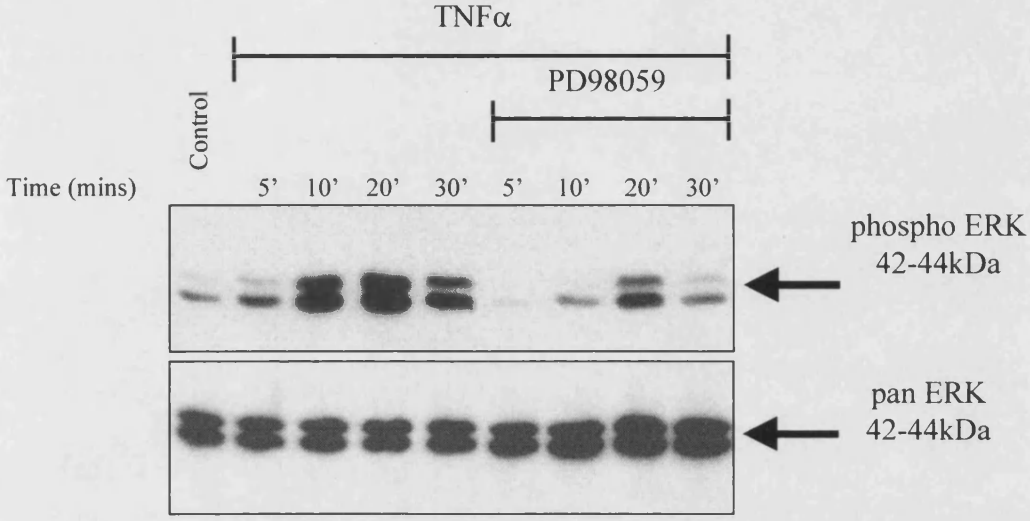


Figure 7.3: ***Inhibition of TNFα induced ERK1/2 activation by PD98059.***
Western analyses of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course in the presence or absence of the specific MEK1/2 inhibitor PD98059 (10μM for one hour) and then probed with a specific antibody against phospho$^{202, 204}$ ERK1/2 (upper panel). The membrane was then stripped and reprobed with the antibody against the unphosphorylated protein to show equal loading (lower panel). Blots are from single experiments but are representative of at least two others.

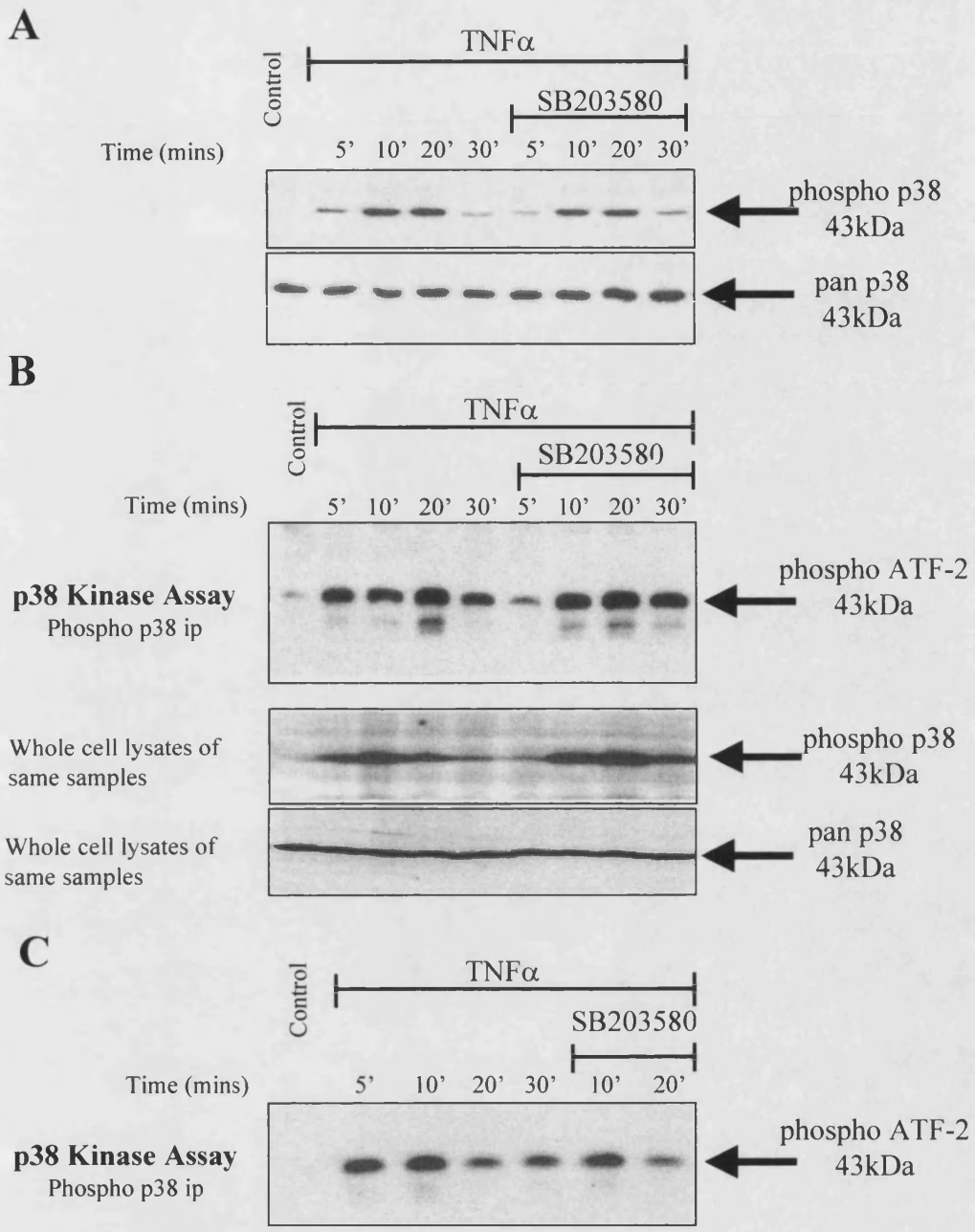


Figure 7.4: ***The action of SB203580 on phosphorylation and in vitro kinase activity of p38.*** **A.** Western analyses of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course in the presence or absence of the specific p38 inhibitor SB203580 (10μM for one hour) and then probed with a specific antibody against phospho$^{180, 182}$ p38 (upper panel). The membrane was then stripped and reprobed with the antibody against the unphosphorylated protein to show equal loading (lower panel). **B.** *In vitro* kinase assay of p38 activity in HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course in the presence or absence of SB203580 (10μM for one hour). Activity of p38 is relative to amount of p38 dependent ATF-2 phosphorylation as shown by the intensity of the phospho^{71} ATF-2 band in the samples immunoprecipitated with p38 (upper panel). The whole cell lysates of the same samples were analysed by western blot and probed for phospho$^{180, 182}$ p38 (middle panel) or unphosphorylated p38 (lower panel) . **C.** *In vitro* kinase assay of p38 activity in HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course in the presence or absence of SB203580 (10μM for one hour) and with SB203580 being again added to the kinase assay after the washing associated with immunoprecipitation. Blots are from single experiments but are representative of at least one other.

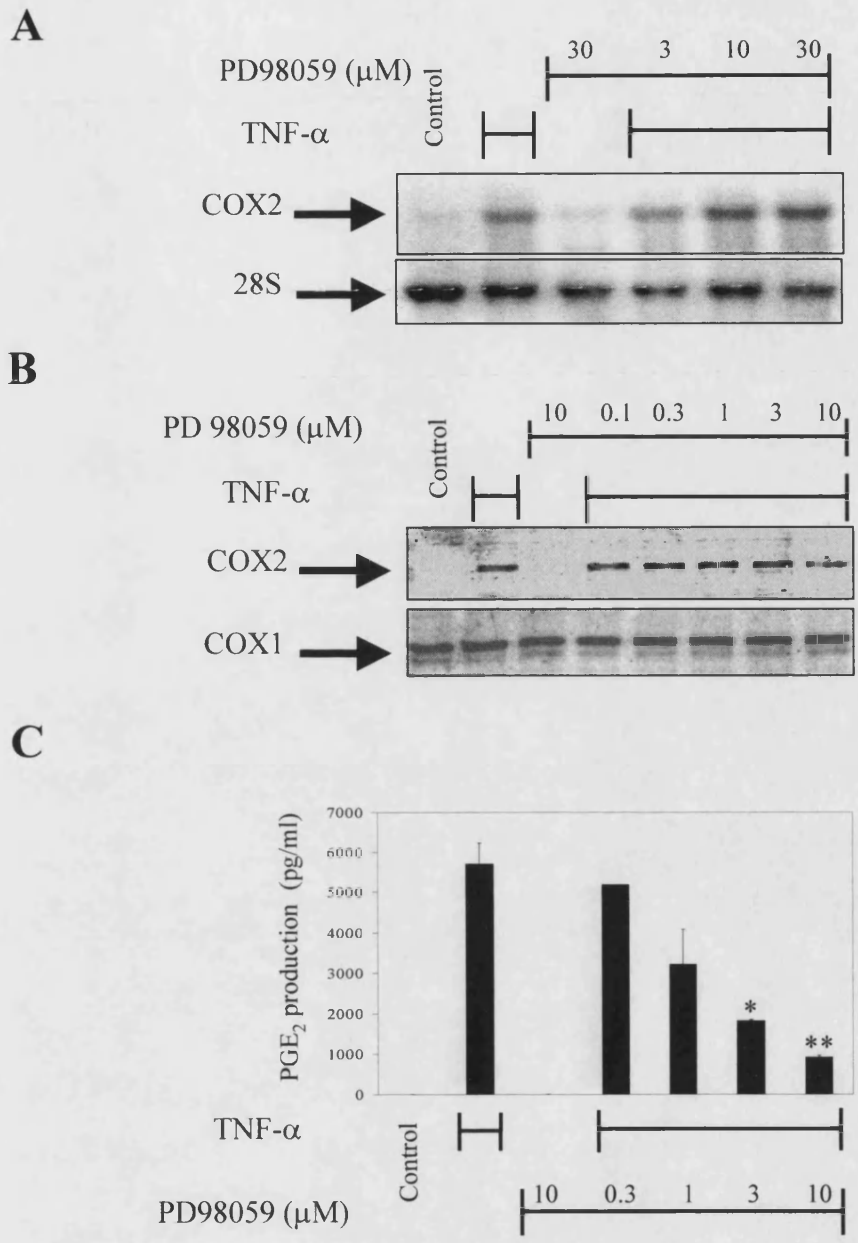


Figure 7.5: ***Regulation of TNFα induced COX2 expression by PD98059.***
A. Northern analyses of mRNA isolated from HT-29 cells, which have been stimulated with TNFα (100ng/ml) for two hours having been pre-treated with increasing concentrations of PD98059 (0-30μM for one hour) and probed for COX2 (upper panel). The 28S band is shown to demonstrate loading (lower panels). **B**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with TNFα (100ng/ml) having been pre-treated with increasing concentrations of PD98059 (0-10μM), and probed with either a specific anti-COX2 (upper panel) or anti-COX1 antibody (lower panel). **D**. An ELISA for PGE_2 (pg/ml) using supernatants from HT-29 cells stimulated with TNFα (100ng/ml) for 24 hours having been pre-treated with increasing concentrations of PD98059 (0-10μM). Significant inhibition of PGE_2 production by additional PD98059 pre-treatment compared to TNFα alone are represented by * ($p<0.05$) and ** ($p<0.01$). This is the result of one experiment using triplicate samples and is representative of two other experiments. Blots are from single experiments but are representative of at least two others. Some of these results were obtained supervising Miss K.M. Patel in her final year project.

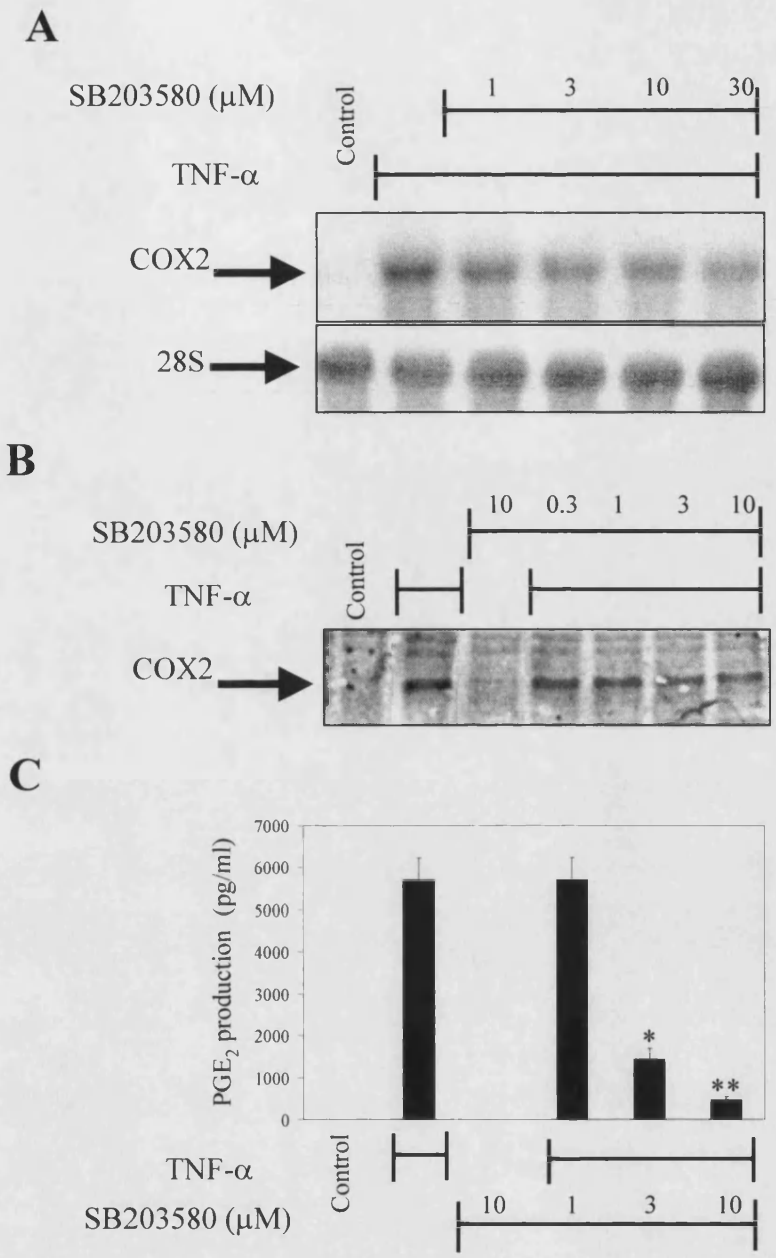


Figure 7.6: ***Regulation of TNFα induced COX2 expression by SB203580.***

A. Northern analyses of mRNA isolated from HT-29 cells, which have been stimulated with TNFα (100ng/ml) for two hours having been pre-treated with increasing concentrations of SB203580 (0-30μM for one hour) and probed for COX2 (upper panel). The 28S band is shown to demonstrate loading (lower panels). **B**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with TNFα (100ng/ml) having been pre-treated with increasing concentrations of SB203580 (0-10μM), and probed with a specific anti-COX2 antibody. **D**. An ELISA for PGE_2 (pg/ml) using supernatants from HT-29 cells stimulated with TNFα (100ng/ml) for 24 hours having been pre-treated with increasing concentrations of SB203580 (0-10μM). Significant inhibition of PGE_2 production by additional SB203580 pre-treatment compared to TNFα alone are represented by * ($p<0.05$) and ** ($p<0.01$). This is the result of one experiment using triplicate samples and is representative of three other experiments. Blots are from single experiments but are representative of at least two others. Some of these results were obtained supervising Miss K.M. Patel in her final year project.

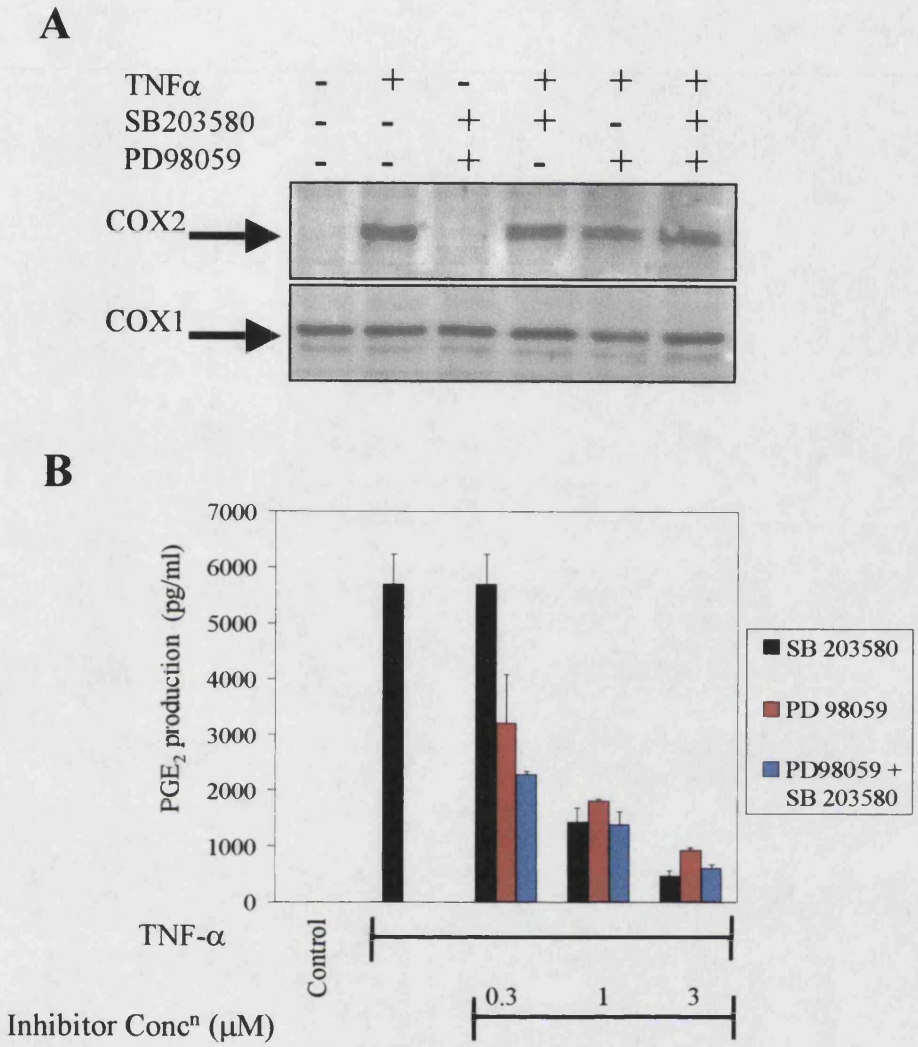


Figure 7.7: ***Regulation of TNFα induced COX2 expression by PD98059 and SB203580.*** **A**. Western analysis of protein isolated from HT-29 cells, stimulated for six hours with TNFα (100ng/ml) having been pre-treated with increasing concentrations of PD98059 (3μM) and SB203580 (3μM) both alone and together, and probed with a specific anti-COX2 antibody (upper panel) or a specific COX1 antibody (lower panel). **B**. An ELISA for PGE_2 (pg/ml) using supernatants from HT-29 cells stimulated with TNFα (100ng/ml) for 24 hours having been pre-treated with increasing concentrations of PD98059 (0-3μM) and SB203580 (0-3μM) both alone and together. This is the result of one experiment using triplicate samples and is representative of two other experiments. Blots are from single experiments but are representative of at least two others.

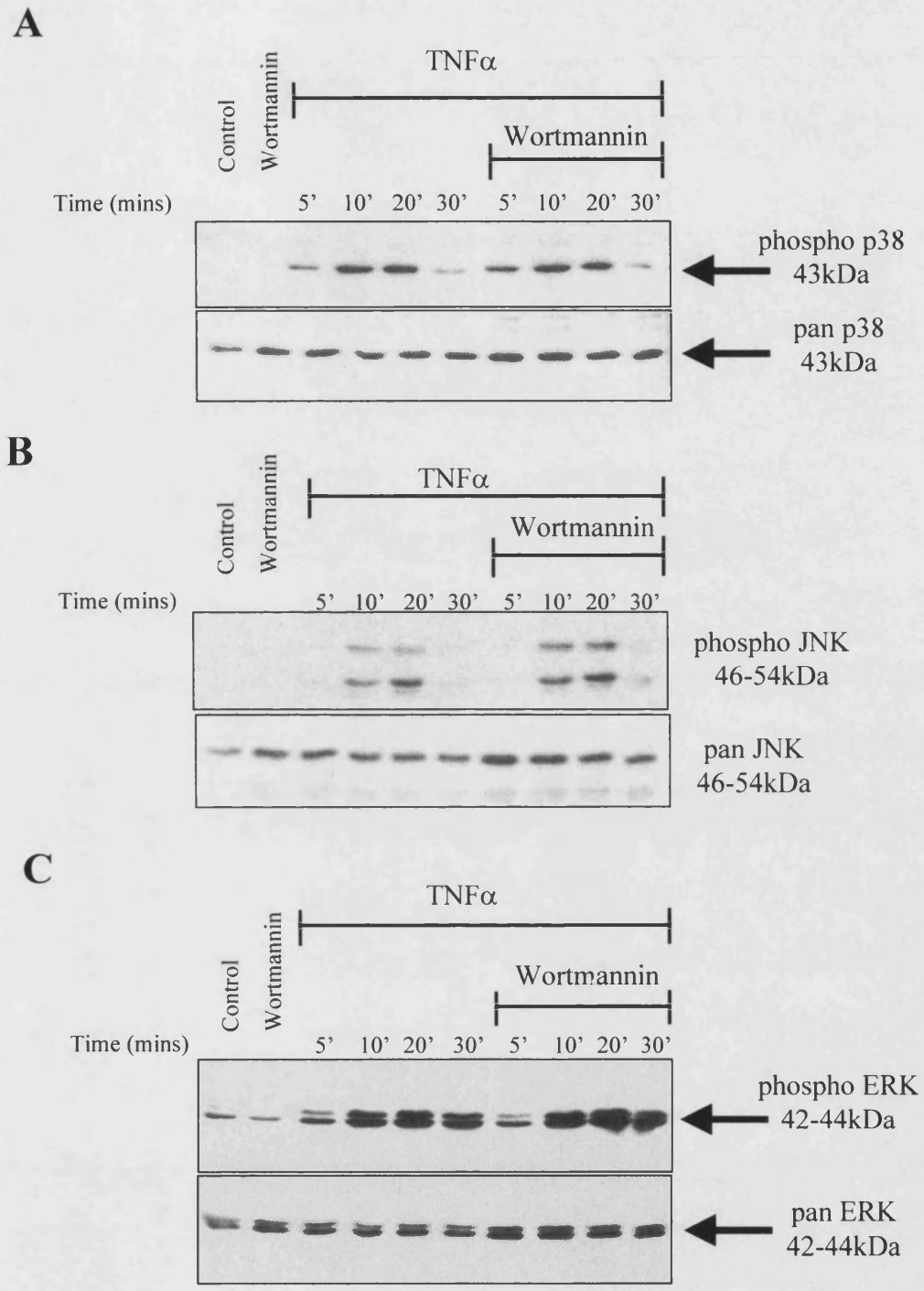


Figure 7.8: ***Activation of MAP Kinases by TNFα is unaltered by PI 3-Kinase inhibition.*** **A to C.** Western analyses of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course in the presence or absence of pre-treatment with the PI 3-Kinase inhibitor wortmannin (100nM for 15 mins) and then probed with specific antibodies against: phospho$^{180,\ 182}$ p38 (**A**), phospho$^{183,\ 185}$ JNK (**B**) or phospho$^{202,\ 204}$ ERK1/2 (**C**) (upper panels). Membranes were then stripped and reprobed with the respective antibodies against the unphosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

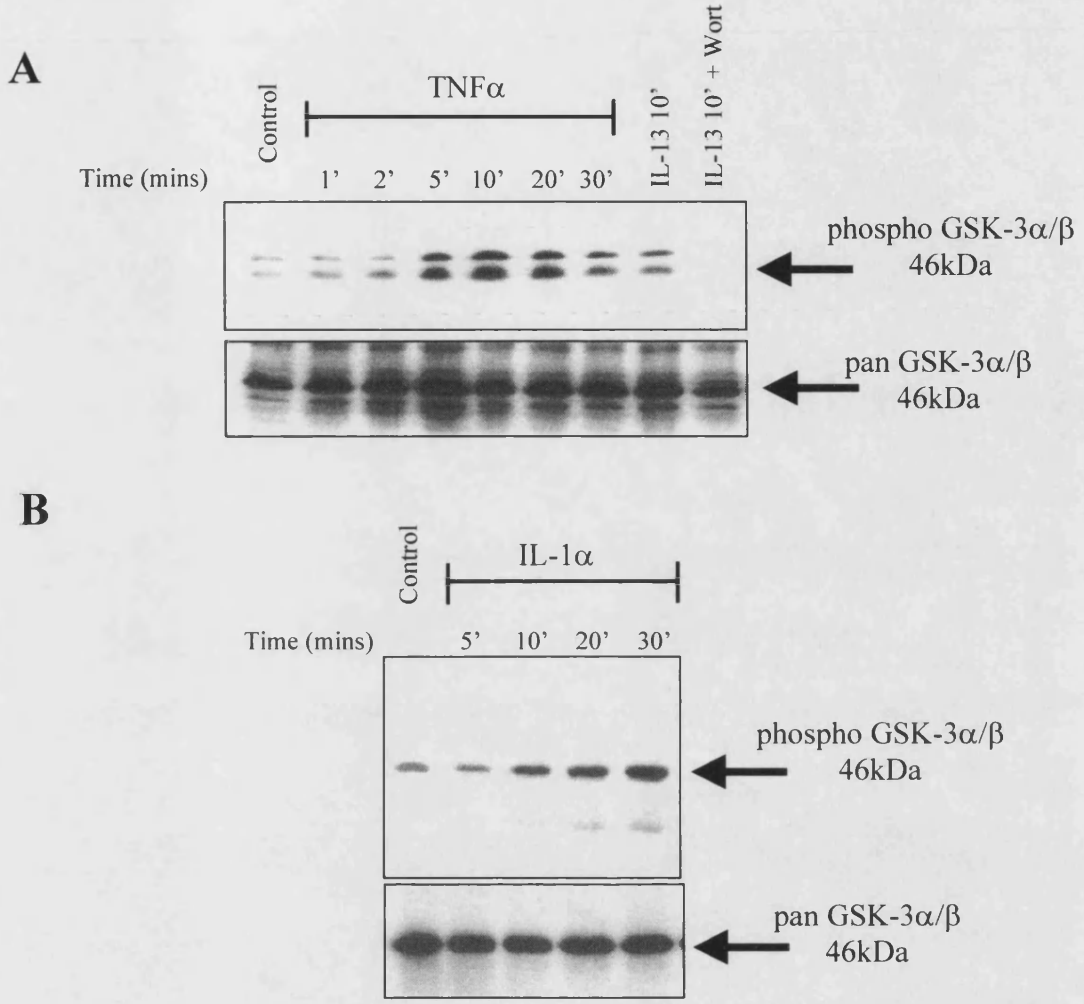


Figure 7.9: ***Activation of GSK-3α/β by TNFα or IL-1α in HT-29 cells.***
A and B. Western analyses of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) (**A**) or IL-1α (10ng/ml) (**B**) over a 30 minute time course and then probed with a specific antibody against phospho[21, 9] GSK-3α/β (upper panels). Membranes were then stripped and reprobed with the respective antibody against the unphosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

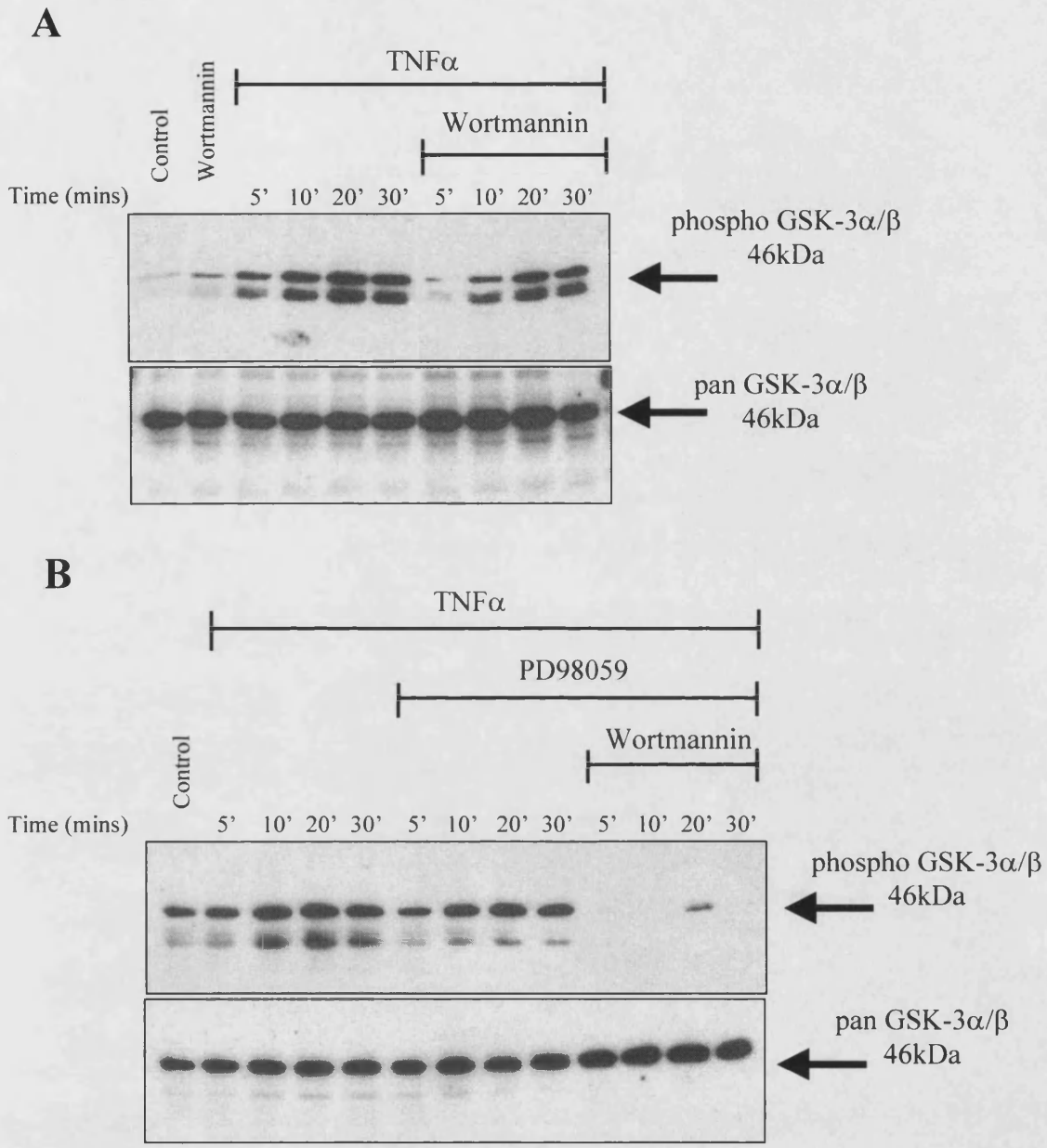


Figure 7.10: ***Combined inhibition of GSK-3α/β by PD98059 and wortmannin***
A and B. Western analyses of protein isolated from HT-29 cells stimulated with TNFα (100ng/ml) over a 30 minute time course in the presence or absence of pre-treatment with the PI 3-Kinase inhibitor wortmannin (100nM for 15 mins) (**A**), or the MEK1/2 inhibitor PD98059 (10μM for one hour) (**B**) or both combined (**B**), and then probed with a specific antibody against phospho[21, 9] GSK-3α/β (upper panels). Membranes were then stripped and reprobed with the respective antibody against the unphosphorylated protein to show equal loading (lower panels). Blots are from single experiments but are representative of at least two others.

7.3 Results Summary

- TNFα and IL-1α induced the phosphorylation and presumed activation of the three MAP Kinases, p38, ERK1/2 and JNK in HT-29 cells.
- PD98059, a specific inhibitor of MEK1/2, the enzymes which activate ERK1/2, inhibited the induced phosphorylation of ERK1/2.
- PD98059 also demonstrated a regulatory role for ERK1/2 on induced COX-2. Incubation with this inhibitor of the ERK pathway caused an up-regulation of induced COX-2 mRNA. In contrast, PD98059 caused an inhibition of induced COX-2 protein and PGE_2 production.
- A specific inhibitor of p38, SB203580, does not prevent the induced phosphorylation of p38. Furthermore, SB203580 did not inhibit p38 activity as measured by an *in vitro* kinase assay.
- Despite its apparent lack of measured inhibition of p38, SB203580 did inhibit induced COX-2 mRNA, protein and PGE_2 production.
- There was no evidence of combinatorial inhibition of COX-2 dependent PGE_2 product using both PD98059 and SB203580.
- Inhibition of PI 3-Kinase with wortmannin did not alter the TNFα induced phosphorylation of p38, ERK1/2 or JNK.
- Both TNFα and IL-1α induced the phosphorylation and presumed inactivation of GSK-3α/β. This could be partially abrogated by inhibition of both PI 3-Kinase and the ERK1/2 pathways although complete inhibition of phosphorylation was only possible using a combination of PI 3-Kinase and ERK1/2 pathway inhibitors.

7.4 Discussion

The work presented here demonstrates that TNFα and IL-1α induce the phosphorylation, and thus the presumed activation, of the three main MAP Kinases, p38, ERK and JNK, in HT-29 intestinal epithelial cells. This is a novel, although not unexpected finding, as pro-inflammatory stimuli such as TNFα and IL-1α are known to induce MAP Kinase activity in a number of systems (Saklatvala *et al.*, 1998; Tibbles and Woodgett, 1999; Cario *et al.*, 2000). The MAP Kinase cascades are activated at times of stress and inflammation, and in this model, TNFα and IL-1α promote an apparently classical inflammatory response. It is worth pointing out the difference in the kinetics of activation of the MAP Kinases by TNFα and IL-1α. IL-1α causes a slightly later and more prolonged phosphorylation of all three MAP Kinases when compared to that seen by TNFα. This highlights the existence of differing pathways downstream of their respective receptors despite the perceived similarity of their induced responses (Saklatvala *et al.*, 1998; Toshina *et al.*, 2000).

Of more interest is the functional outcome of MAP Kinase activation with regards to COX-2 expression in this system. To investigate this, well known inhibitors of the ERK signalling pathway (PD98059) and the p38 pathway (SB203580) were used. PD98059 is an inhibitor of MEK1/2 which is the kinase upstream of ERK1/2 and which activates it by catalysing its phosphorylation. It is regarded as a specific inhibitor (Davies *et al.*, 2000) and in this work behaved in a predictable way. Pre-incubation with 10μM PD98059 significantly abrogated the TNFα induced

phosphorylation of ERK1/2 (Figure 7.3) as would be expected. The results with the inhibitor of the p38 pathway were more complex.

SB203580 is a pyridinyl imidazole compound which inhibits p38 by competitively binding to the ATP binding site (Young *et al.*, 1997; Cuenda *et al.*, 1995). It is termed a specific inhibitor although there is evidence that it can inhibit lymphocyte kinase and GSK-3β, although at IC_{50}s two orders of magnitude higher than their effects on p38 (Davies *et al.*, 2000). Of greater relevance are reports demonstrating the inhibition of PKBα (Davies *et al.*, 2000; Lali *et al.*, 2000) and some members of the JNK family (Dean *et al.*, 1999) at concentrations similar to those needed to inhibit p38. Such findings make it necessary to interpret with care results using this compound.

A second note of caution concerns the mechanism of action of SB203580. Most papers just use the assumption that SB203580 is a specific p38 inhibitor, but the literature reveals heterogeneity in the results of a variety of assays used to document its inhibition of p38 when this is assessed (see Table 8.1). Firstly, regarding using phosphorylation of p38 as a marker of its activity, as SB203580 binds to the separate ATP binding site on p38 and not to its phosphorylation site, there is no apparent reason why it could not bind and prevent activity without preventing phosphorylation. Such a mechanism would support the results presented here where SB203580 does not alter phosphorylation of p38 at a concentration subsequently shown to inhibit COX-2 expression and PGE_2 production. However, although there is support for this hypothesis in the literature (Cuenda *et al.*, 1995; Young *et al.*, 1997), it is not universal (Frantz *et al.*, 1998).

Effect of SB203580 on marker of p38 pathway activity	**Frantz** (Frantz *et al.*, 1998)	**Cuenda** (Cuenda *et al.*, 1995)	**Young** (Young *et al.*, 1997)	**Dean** (Dean *et al.*, 1999)	***Weaver***
Phosphorylation of p38	**Yes**	**No**	**No**		***No***
p38 Kinase assay *Phosphorylation of MAPKAP-2*		**Yes**	**No**		
ATF-2	**Yes**				***No***
MAPKAP-2 Kinase assay *Phosphorylation of hsp27*		**Yes**	**Yes**	**Yes**	
Inhibition of "p38 dependent" event	**Yes**	**Yes**		**Yes**	***Yes***

Table 8.1: ***Table demonstrating the heterogeneity of results concerning the effect of the p38 inhibitor, SB203580, on markers of p38 activation***

The second potential marker of p38 activity is a direct *in vitro* kinase assay employing a specific action of p38. Two assays are commonly used; the phosphorylation and activation of MAPKAP2, and the phosphorylation and activation of ATF-2. Again there is heterogeneity of the published results – see Table 8.1. Young *et al* found that SB203580 pre-incubation did not inhibit p38 kinase activity, although the addition of the compound to the kinase assay after the necessary washes did cause inhibition (Young *et al.*, 1997). In the work here, neither

pre-incubation with SB203580 alone, or pre-incubation with subsequent repeated addition of SB203580 to the kinase assay resulted in inhibition.

The third potential marker of p38 activity was phosphorylation of hsp27, a reaction catalysed by MAPKAP2 which in turn has been activated by p38. In all instances this demonstrated inhibition by SB203580, even in conditions of sole pre-incubation with SB203580 and no subsequent addition to the kinase assay reaction mixture, conditions which had shown apparent absence of inhibition of p38 kinase activity (Young *et al.*, 1997; Paul *et al.*, 1999).

Why is there such heterogeneity in results concerning p38 activity? Primarily, it is apparent that experimental conditions and techniques, as well assays used, make a significant difference to the results obtained. SB203580 is a competitive inhibitor which can be washed off. Therefore, in kinase assays, it may need to be added to the final reaction in some, although not all, circumstances. In these kinase assays, the broken cell lysates may not represent accurately the normal physiological function of the cell, with the natural *milieu* of the cell being significantly altered by the phosphatase inhibitors in lysis buffer. Secondly, there is some general consensus, and mechanistic logic, that the phosphorylation state of p38 is not necessarily altered by effective SB203580 binding.

In this work, SB203580 caused inhibition of COX-2 expression and marked inhibition of PGE_2 production although inhibition of p38 activity could not be demonstrated. This was not due to the SB203580 vehicle (DMSO) (Figures 5.2, 5.4C and 5.6) but may be due to other actions of SB203580. However, there is

evidence to support the statement that SB203580 was still likely to be inhibiting p38, despite that not being clearly demonstrated, because of the technicalities of the assays used.

As already mentioned, both p38 inhibition with SB203580 and ERK1/2 inhibition with PD98059, altered COX-2 expression and functional activity. Starting with the actions of presumed p38 inhibition by pre-incubation with SB203580, this caused a minor concentration dependent inhibition of TNFα induced COX-2 mRNA and protein. It did however cause a significant inhibition of TNFα induced PGE_2 production with an IC_{50} of 1.5μM. This is in agreement with other published work showing that cytokine induced COX-2 expression was decreased by p38 inhibition in renal (Cheng *et al.*, 2000), HeLa (Ridley *et al.*, 1998), macrophage (Hwang *et al.*, 1997; Paul *et al.*, 1999) and cardiac (LaPointe and Isenovic, 1999) cell lines. The site of this regulatory effect has been investigated with it being demonstrated that, for IL-1 stimulation of HeLa cells, SB203580 inhibits induced PGE_2 production but not induced arachidonic acid release, implying a COX-2 dependent mechanism (Ridley *et al.*, 1998). This group have gone on to show that p38 dependent pathways regulate COX-2 at the point of its mRNA stability in their model (Ridley *et al.*, 1998; Lasa *et al.*, 2000). Indeed, they demonstrate that a 123 nucleotide fragment of the COX-2 3′-UTR was necessary and sufficient for this regulation and identified a protein similar to the AU-rich element/poly(U) binding factor 1 (AUF-1) (Lasa *et al.*, 2000). Such a mechanistic action of p38 inhibition would explain the observation that SB203580 can inhibit cytokine induced COX-2 expression but over-expression of p38 alone does not cause COX-2 induction as, in the absence of constitutive expression, there would be no COX-2 mRNA to stabilise (Hwang *et al.*, 1997). It

also provides a mechanism for the well documented inhibition of COX-2 by dexamethasone (Lasa *et al.*, 2001).

It was shown in chapter 6 how wortmannin can regulate COX-2 using mRNA stability in this intestinal epithelial cell system. However, in that case and in the work cited here (Ridley *et al.*, 1998; Lasa *et al.*, 2000), the responses to the regulatory element (wortmannin and SB203580 respectively) showed similar concentration dependent reponses for the actions on COX-2 mRNA, protein and PGE_2 production. For the regulatory action of SB203580 on TNFα induced COX-2 in HT-29 cells, the inhibition is much more marked at the level of PGE_2 production. This implies a predominantly post-translational effect of SB203580, while not ruling out an alteration of COX-2 mRNA stability to account for the slight decrease seen in COX-2 mRNA at higher concentrations of SB203580. Although there are many possible sites of potential post-translational regulation, one proposed by LaPointe *et al* is that of substrate availability (LaPointe and Isenovic, 1999). They demonstrated that IL-1β induces both COX-2 and $cPLA_2$ in cardiac myocytes and that the latter is also inhibited by p38 inhibition. This would potentially decrease the substrate pool available for the induced COX-2 resulting in a disproportionate decrease in PGE_2 production compared to the amount of COX-2 protein present on western blot analysis. These workers make a similar point for ERK1/2 inhibition with PD98059 which would also seem appropriate for the results presented here.

Regarding ERK1/2 inhibition, there is generally less literature than that with p38 inhibition. This is probably because p38 represents a better potential anti-inflammatory target for new drugs than does ERK1/2 which is intrinsically involved

in cell proliferation (Saklatvala *et al.*, 1998; Herlaar and Brown, 1999). However there is evidence that ERK1/2 inhibition does inhibit COX-2 expression and functional activity (Hwang *et al.*, 1997; LaPointe and Isenovic, 1999). This work has similar results as for p38 inhibition, with PD98059 having a marked effect at the level of PGE_2 production which seems disproportionate to its effects on COX-2 protein and implies a post-translational mechanism. Furthermore, there is a paradoxical increase in TNFα induced COX-2 mRNA, similar to the opposing effects of IFNγ on COX-2 at mRNA as opposed to protein and product levels (chapter 5).

Finally, with respect to the use of ERK1/2 and p38 inhibitors, there is evidence in the literature that there can be a combinatorial effect with their use (LaPointe and Isenovic, 1999; Rutault *et al.*, 2001). However, in this work, such effects were not observed. This is somewhat surprising as it might be expected if the MAP Kinase pahways were running in parallel as is classically proposed.

Regarding the results investigating the effect of PI 3-Kinase inhibition and MAP Kinase activation, there were two hypotheses being considered. Firstly, there is evidence in some, but not all cell lines, that MAP Kinase activation is PI 3-Kinase dependent (Jiang *et al.*, 2000; Yamaki *et al.*, 2000; Yamboliev *et al.*, 2000). In this case wortmannin would be expected to inhibit TNFα induced MAP Kinase activation. Alternatively, if the effect of wortmannin at increasing COX-2 expression and activity is mediated via a MAP Kinase dependent pathway, it would be expected that wortmannin would potentiate the activation of MAP Kinases by TNFα. The results presented here demonstrate no alteration of TNFα induced MAP

Kinase activation in the presence of pre-incubation with wortmannin. This would apparantly negate both hypotheses and implies that, in HT-29 intestinal epithelial cells, PI 3-Kinase and MAP Kinase pathways are running parallel to each other. It also seems unlikely that PI 3-Kinase activation is MAP Kinase dependent as PI 3-Kinase activation is seen within 30 seconds of TNFα stimulation. This would be supported by other work which always shows PI 3-Kinase to be upstream in models where MAP Kinase is PI 3-Kinase dependent with no evidence of the converse relationship.

The final piece of work in this chapter concerns the phosphorylation and inactivation of GSK-3α/β by TNFα and the signalling pathways involved. As mentioned in the introduction, the GSK signal is involved in colorectal carcinogenesis where it is an anti-oncogenic signal resulting in β-catenin degradation as part of the Wnt pathway. GSK is inactivated by phosphorylation and this phosphorylation has been shown to be PI 3-Kinase dependent (Cross *et al.*, 1995), thus further supporting the pro-proliferative actions of PI 3-Kinase (Roymans and Slegers, 2001). Figure 7.9 demonstrates that TNFα and IL-1α phosphorylate GSK-3α/β in a time dependent manner with the phosphorylation caused by IL-1α being more prolonged, as seen with the MAP Kinases. IL-13, which is known to activate PI 3-Kinase in HT-29 cells, acts as a wortmannin sensitive positive control (Wright *et al.*, 1997). Interestingly, wortmannin only caused a partial abrogation of the TNFα induced GSK-3α/β phosphorylation, which was surprising and implied a second pathway mediating the TNFα signal. Work using the ERK1/2 inhibitor PD98059 showed that it too caused a partial abrogation of the GSK-3α/β phosphorylation caused by TNFα. When combined, TNFα induced GSK-3α/β phosphorylation was completely

inhibited. This implies that this process is dependent on PI 3-Kinase and ERK1/2 dependent signals which run in parallel. This supports the previous conclusion that the MAP Kinases, and in this case ERK1/2, are not downstream of PI 3-Kinase but are independent signalling pathways in this context. Furthermore it highlights a role for ERK1/2 in GSK-3α/β inactivation by phosphorylation which has not previously been demonstrated. This is, however, perhaps unsurprising given the known functional role of ERK1/2 in proliferation.

8 General Discussion

A number of general points are briefly made below concerning the work presented here. They represent general issues raised by the work, rather than specific points which have already been discussed.

8.1 Experimental Model

The experimental model used in this work was *in vitro* culture of transformed intestinal epithelial cell lines and the assessment of pro-inflammatory cytokine induction of COX-2 expression as well as intra-cellular signalling responses. In this context COX-2 appears to be behaving as an acute pro-inflammatory agent – induced by pro-inflammatory cytokines, inhibited by Th2 immunoregulatory cytokines and having its expression regulated by the MAP Kinases and NFκB. Although allowing detailed manipulation of the regulatory pathways involved, the experimental model lacks the ability to pursue the more complex points concerning COX-2 such as a potential anti-inflammatory role in chronic disease. The system may also over-estimate the signalling pathways activated by TNFα and IL-1α as transformed cell lines are believed to be more sensitive for activation of signalling cascades (Saklatvala *et al.*, 1998).

8.2 Drawing Conclusions and Making Comparisons

One feature of the literature that is obvious is the heterogeneity in results between different models and cell types. It is therefore important that, although much can be

learnt from work in other systems, results must be confirmed in a system relevant to the physiological system of interest – in this case the gastro-intestinal tract. Furthermore, because of the limitations of transformed cell lines, albeit human ones, work should ideally be repeated and validated in similar systems. There may be a common theme in the literature, such as TNFα or IL-1α inducing COX-2 which is shared in many systems. Other points, such as the regulatory actions of IFNγ, are extremely system dependent. The conclusions outlined in Figure 8.1 are therefore from this work and similar work in gastro-intestinal systems, rather than using other models which may not be relevant.

On a similar note, conclusions from this work are strongest when using a variety of techniques, such as the TNFα activation of PI 3-Kinase, or when consistent results are obtained using similar inhibitors. As is seen with the PI 3-Kinase inhibitors, wortmannin and LY294002, this is not always the case making the drawing of clear conclusions more difficult.

8.3 Complex Interactions of Signalling Pathways

As seen in Figure 8.1 there are many regulatory elements in an important gene such as COX-2 with the possibility that there may be interactions between them. There is also an element of redundancy and the possibility that stimulation may activate opposing signals. Such complexities make drawing detailed conclusions difficult. They also make the teasing out of the individual pathway effects challenging and often limited by the available molecular tools.

8.4 Similarities Between TNFα and IL-1α

In all of the work presented here, TNFα and IL-1α have the same biological outcome, albeit at times with differing kinetics. However in human disease the targeting of TNFα for therapies has proved far more successful than targeting IL-1α and the reasons for this have not been particularly clear. The two cytokines may share targets, but have differing pathways for activating them, and thus the reason for one being a superior therapeutic target may lie in the complexities of intra-cellular signalling.

8.5 Application to Gastroenterological Diseases

In the detailed discussions associated with each chapter there has been little comment on the implications for gastro-intestinal diseases. This is partly because work in such a model is quite separated from the complex physiological systems in the human body. However there are some general points that can be made:

1 Firstly, in acute inflammation all the results here continue to support a pro-inflammatory role for COX-2 in the gut although this may not be true for chronic inflammation.

2 Secondly, as it is likely that COX-2 inhibition is likely to be beneficial in the chemoprevention of colorectal carcinoma, this work identifies more potential methods of COX-2 inhibition including the p38 and ERK1/2 MAP Kinases.

3 Thirdly, from the point of view of possible therapeutic targets of the future, there is the question of specificity. Basic science research, when applied to a clinical speciality such as gastroenterology, often states as its aims the gaining of a more precise understanding of molecular pathophysiology and

the subsequent development of specific therapeutic modalities. Although the former is most definitely true, the latter point needs to be developed. Therapy involving inhibition of TNFα in Crohn's disease is regarded as a specific molecular therapy. However, it can be seen here that TNFα results in the activation of multiple and potentially conflicting pathways in the intestinal epithelium alone, disregarding the infiltrating immune cells seen in IBD. Such therapies are not as isolated and precise as is often stated and it is worth pointing out that drugs such as 5-ASA, with multiple and not fully understood mechanisms of action, still provide a mainstay of management of IBD. Thus, although it is vital to fully understand the molecular processes of disease, it is not always necessary for a therapy to be precise with only one mechanism of action.

4 The COX pathway is highly complex with multiple points of external and auto-regulation. Downstream prostaglandins, as well as upstream arachidonic acid liberation by phospholipases, will play a physiological role. Thus, isolating the precise function of COX expression in complex physiological systems will be a challenge which will continue into the future.

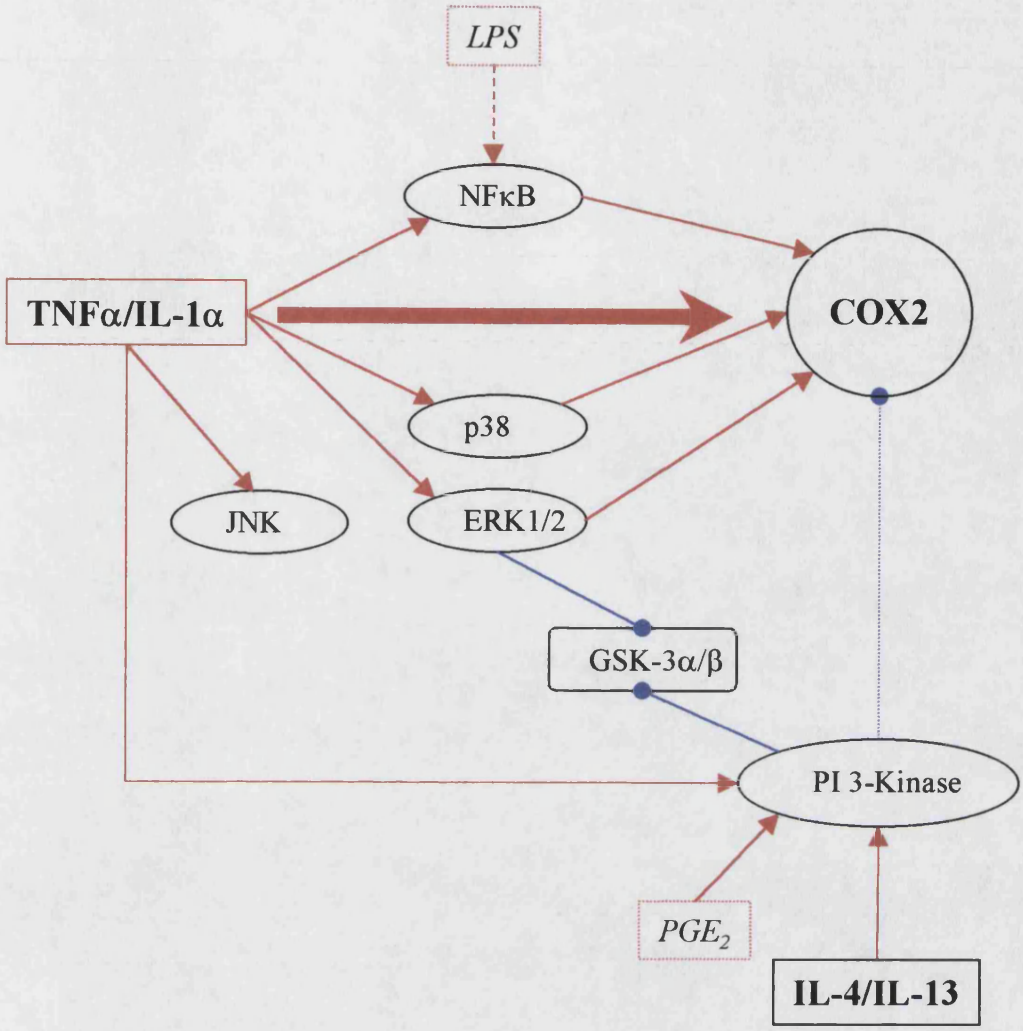


Figure 8.1: ***Schematic representation of results concerning COX2 regulation in intestinal epithelial cells***

This schema draws predominantly on results from this work with relevant other results from other work being in dotted boxes. Red represents a positive activation and blue an inhibitory inactivation.

9 Conclusions

- COX-2 can be induced in intestinal epithelial cells by the pro-inflammatory cytokines TNFα and IL-1 α or β
- This induction is regulated at many points including transcription and stability of mRNA, translation into protein and subsequent activity, and the prostaglandins produced. Regulation at these different points may be contrasting at each level.
- The products of COX-2 regulate its expression in opposing ways, as can IFNγ.
- Th2 cytokines, IL-4 and IL-13, down-regulate induced COX-2 as well as activating PI 3-Kinase
- PI 3-Kinase has a regulatory role on induced COX-2, which appears to be inhibitory, although the evidence is conflicting.
- There is strong evidence that TNFα activates PI 3-Kinase in intestinal epithelial cells. This is supported by data that IL-1α has a similar action.
- Both TNFα and IL-1α also activate the three major MAP Kinases in a PI 3-Kinase independent manner. They both also inactivate GSK-3α/β which is partially sensitive to PI 3-Kinase inhibition or ERK1/2 inhibition and completely inhibited when these inhibitors are used in combination.
- ERK1/2 and p38 have a post-transcriptional inhibitory role on induced COX-2 expression.
- In intestinal epithelial cells the activation of multiple signalling pathways by Th1 cytokines, and the complex regulation of induced COX-2, have important implications for gastro-intestinal disease which are only beginning to be understood.

10 Future Work

This work has been fortunate to coincide with a great expansion of interest in intracellular signalling in clinically relevant conditions. In making some novel findings regarding the role of signalling pathways, and PI 3-Kinase in particular, as well as pointing out the complexities of COX-2 expression and regulation within the gastrointestinal tract, it mirrors reasonably accurately the current state of this area of research. However, there is much to be done to convert these initial findings in a simple system into a more cogent understanding of the role of signalling and COX-2 expression in gastrointestinal physiology and disease. This includes:

- Persevering with methods to study COX-2 expression, and the activation of signalling pathways, in fresh human tissue as opposed to transformed cell lines.
- Supporting the work presented here with work in other models using other techniques. In particular, regarding the work based on PI 3-Kinase inhibition, other techniques should be used such as molecular inhibition using dominant negative or constitutively active transfected constructs.
- This area of COX-2 research concentrates on the enzyme rather than its products, which are the focus of its functional activity. Prostaglandins, and their differential synthesis and regulation, warrant more research.
- Models need to be developed which differentiate acute inflammation from chronic inflammation, and these need to be specific to the gut. It is highly likely that the functional role of COX-2 in the acute and chronic situation is different.
- Clinical research gastroenterologists need to be aware of research into intracellular signalling as it is likely to provide future therapeutic options, albeit not necessarily a "magic bullet", in IBD and colorectal carcinoma.

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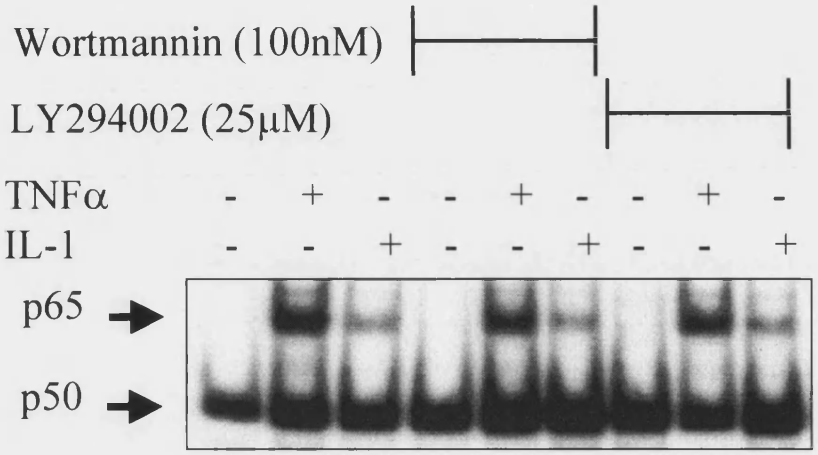

Appendix A: ***Cytokine induced NFκB binding activity is not altered by PI 3-Kinase inhibition***

HT-29 cells were stimulated with TNFα or IL-1 (both at 5ng/ml for 30 mins) with or without 30 mins pre-treatment with two independent PI 3-Kinase inhibitors, wortmannin (100nM) or LY294002 (25μM). Nuclear extracts (5μg) were tested for NFκB binding activity by EMSA. Pre-treatment with PI 3-Kinase inhibitors did not alter cytokine induced activation of NFκB binding activity. Blot is from a single experiment but is representative of at least three others. This work was carried out by Maria Pia Russo and Christian Jobin at the University of North Carolina, Chapel Hill as part of a collaborative study.